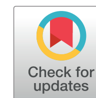

Pancreatic Pseudoislets: An Organoid Archetype for Metabolism Research

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Pancreatic islets are vital endocrine regulators of systemic metabolism, and recent investigations have increasingly focused on understanding human islet biology. Studies of isolated human islets have advanced understanding of the development, function, and regulation of cells comprising islets, especially pancreatic α- and β-cells. However, the multicellularity of the intact islet has stymied specific experimental approaches—particularly in genetics and cell signaling interrogation. This barrier has been circumvented by the observation that islet cells can survive dispersion and reaggregate to form "pseudoislets," organoids that retain crucial physiological functions, including regulated insulin and glucagon secretion. Recently, exciting advances in the use of pseudoislets for genetics, genomics, islet cell transplantation, and studies of intraislet signaling and islet cell interactions have been reported by investigators worldwide. Here we review molecular and cellular mechanisms thought to promote islet cell reaggregation, summarize methods that optimize pseudoislet development, and detail recent insights about human islet biology from genetic and transplantation-based pseudoislet experiments. Owing to robust, international programs for procuring primary human pancreata, pseudoislets should serve as both a durable paradigm for primary organoid studies and as an engine of discovery for islet biology, diabetes, and metabolism research.

Diabetes pathogenesis involves multiple mechanisms, reflected by a spectrum of manifestations, including type 1 diabetes, type 2 diabetes (T2D), and type 3c diabetes (1). Underlying this diabetes diversity is the unifying concept that pathology and dysfunction of the pancreatic islets of Langerhans are common to all types of diabetes. Thus, intensive efforts in diabetes research are focused on understanding of islet biology, genetics, signaling, and function in physiological or pathophysiological settings, framed by the goal of generating targeted therapies responsive to each form of diabetes.

Increasingly, these efforts have focused on investigations of human islets, which have multiple properties distinct from rodent islets: these include different endocrine cell composition and arrangement; physiological differences, including differences in glucose set point and basal and stimulated insulin and glucagon secretion (2,3); and molecular differences, including distinct transcriptomic, epigenomic, and chromatin signatures (4,5). Although the mechanism(s) underlying these species differences is not entirely clear, prior studies suggest that the predominance of heterologous contacts between α- and β-cells enhances regulation of insulin secretion by glucagon in human islets, compared with rodent islets (6). These specific features motivate studies of human islets.

Pancreatic islet architecture is complex, comprised of interspersed endocrine α-, β-, δ-, ε-, and PP islet cells comingled with multiple nonislet cell types, including endothelial cells, stellate cells, smooth muscle, fibroblasts, and resident immune cells (7). Pancreatic islets offer a strikingly broad set of experimental opportunities, stemming from the ability to isolate and purify them from the pancreas of multiple species, including humans. Once isolated, islets can be maintained in vitro with established culture regimes, permitting a range of phenotyping, including functional studies of insulin secretion by β-cells and glucagon secretion by α-cells. Unlike for most other

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organs and tissues, however, islet function can be assessed following transplantation to suitable animal hosts in experimentally convenient, heterotopic engraftment sites, like the subcapsular renal space (8–15). Transplanted islets revascularize and reacquire endocrine functions, including regulated insulin or glucagon secretion stimulated by physiological secretagogues like glucose or amino acids (13).

The impermeability of intact cultured islets to many experimental reagents presents an experimental challenge, especially for genetics. For example, exposure of intact islets to compounds or transgene vectors often leads to changes only in the most superficial islet cell layer, and failure to penetrate the innermost islet regions, limiting or precluding interpretation. To circumvent this, islets can be dispersed into single-cell suspensions and then reaggregated to form multicellular structures. This process of dispersion and reaggregation to form "pseudoislets" was first reported with canine islets (16), and subsequent work showed the feasibility of pseudoislet formation from diverse sources, including rodents, pigs, and humans (17–19). Remarkably, multiple studies have shown that pseudoislets retain the principal islet cell composition and endocrine functions of intact islets. For example, human pseudoislets reform gap junctions linking islet cells (20) and retain glucose-stimulated insulin secretion (GSIS) both in vitro (19,21) and in vivo after transplantation (8). This singular feature of islets among mature solid organs—tolerance of a transient dispersed state—provides powerful experimental opportunities to modulate the composition and genetics of islet cells prior to reaggregation into pseudoislets.

Here we briefly discuss molecular and cellular mechanisms underlying islet cell reaggregation, summarize methods that optimize pseudoislet formation, and detail recent insights about human islet biology from genetic and transplantation-based pseudoislet experiments. These investigations of gene expression, hormone production, stimulus-secretion coupling, and islet cell functional maturation capitalize on the multitude of unique experimental advantages for islet studies. Concurrent advances in genetics, genomics, and transplantation techniques have made it possible to use pseudoislets for interrogation of islet genetics, intraislet signaling, and islet cell interactions, studies less feasible with intact islets, in vitro cell lines, or transgenic mice. The advantages afforded by pseudoislet biology are demonstrated by a surge of recent studies from investigators worldwide.

Mechanisms Guiding Pseudoislet Formation

Cellular dissociation, reaggregation, and self-organization in vitro were the focus of classical studies of the 19th and early 20th centuries and helped to inspire studies of islet self-(re)assembly (16). The process of self-organization has been described in normal developmental processes, wound healing, regeneration, and pathologies in mammalian tissues. Strikingly, dissociated cells from vertebrate embryonic tissues display a propensity to reaggregate and reconstitute multicellular systems (22). Steinberg hypothesized that this self-organization reflects cellular rearrangement toward a thermodynamic equilibrium. Specifically, unbound adhesion sites on the surface of cells represent free energy, and reduction of unbound surface area via binding at the junction between two cells moves the system toward an energy minimum. Based on observations that "like" cells in his studies adhered more strongly to each other than "unlike cells," Steinberg postulated the existence of selective and differential adhesion mechanisms (22). Investigations have since identified specific adhesion molecules that drive self-organization. While the scope of our article precludes an in-depth discussion of this field, we highlight several studies of these processes in the developing pancreas and intact postnatal islets that have improved the understanding and development of pseudoislets.

Cell-cell aggregation is driven primarily by calcium-dependent and calcium-independent cellular adhesion molecules (CAMs) displayed on the surfaces of vertebrate and mammalian cells (23). Among the best characterized CAMs in islets are members of the calcium-dependent cadherin superfamily, epithelial (E-) and neural (N-) cadherin. E-cadherin and N-cadherin are essential for embryonic clustering of pancreatic endocrine cells into islets and are expressed on the surface of mature pancreatic islet cells (24). These transmembrane proteins have an extracellular domain that facilitates cell-to-cell adhesions by forming homodimers with cadherins on neighboring cells and have a cytoplasmic domain that binds to intracellular α- and β-catenins, which bridge to the F-actin cytoskeleton (23). Calcium binding causes conformational changes in these cadherins that decrease their susceptibility to proteolytic cleavage and enable their presentation to mediate islet cell clustering (24). Additionally, E- and N-cadherin may directly regulate insulin secretion in response to glucose, proliferation, and cell viability (25,26).

Calcium-independent CAMs are also present on islet cells and play crucial roles in islet cell clustering and organization. Such CAMs include immunoglobulins, integrins, and claudins (27–29). Neural CAM (NCAM) is one example of a calcium-independent immunoglobulin expressed in islet cells. NCAM expression becomes concentrated in islet cells during pancreatic ontogeny and mediates both homophilic and heterophilic cell-cell interactions. Such interactions are necessary for proper cadherin clustering and islet cell type segregation in development (27).

Gap junctions are another critical element in guiding the aggregation, adhesion, and proper function of islet endocrine cells. The expression and roles of gap junction proteins in endocrine secretion have been extensively reviewed by Meda and others (30). Briefly, these intercellular channels, composed of 12 connexin subunits, are crucial for shuttling ions, metabolites, and other small

molecules between cells (31). In islets, these gap junctions are composed of connexin36 (Cx36) and regulate electrical activity and insulin secretion between β-cells (30). Other connexin species have also been identified at the transcriptome level, though their roles are much less understood (32). These studies have been complemented by those of connexin regulators, such as the EphA receptor tyrosine kinases, and their role in β-cell bidirectional cell-cell communication for proper insulin secretion (33).

The in vitro aggregation of pseudoislets is likely modulated by the same native adhesion molecules governing in vivo islet cell aggregation. Expression of E-cadherin is increased in MIN6 cell pseudoislets relative to monolayers (26,34), and E-cadherin expression correlates with glucose-regulated insulin secretion (35). Additionally, studies in rat pseudoislets demonstrate that E-cadherin is necessary for proper islet cell organization (24) and that differences in NCAM expression between islet cell types also contribute to cell localization within multicellular clusters (36). Murine studies revealed that β-cells promote pseudoislet reaggregation: cultures of mouse α-cells alone or δ-cells alone failed to form pseudoislets, indicating that β-cells may provide key cell-to-cell contacts that drive islet cell clustering (37). Additionally, lentiviral vector targeting of specific connexins demonstrated that modulation of gap junction proteins negatively affected insulin secretion, indicating that appropriate construction of gap junctions between islet cells is critical to proper function (20).

Methods for Human Pseudoislet Formation

Distinct methods have capitalized on the intrinsic islet cell-cell adhesion processes to promote formation of organoid-like multicellular pseudoislet clusters from dispersed human islet cells. To date, well-characterized approaches for achieving human pseudoislet formation include spontaneous, hanging-drop, and microwell reaggregation techniques (summarized in Table 1).

Spontaneous reaggregation is the most accessible and least laborious approach for formation of human pseudoislets. After dissociation, islet cells are introduced to ultra-low attachment plates and reaggregate stochastically into pseudoislets over 5–7 days (see Fig. 1). Approximately one pseudoislet is generated per 5–10 input human islet equivalents (38 and R.J.B., S.K.K., unpublished data). While this approach typically yields pseudoislets that are heterogeneous in size, greater homogeneity can be achieved by reduction of the volume of culture media and by use of gentle centrifugation to prompt clustering (38). Our group and others have demonstrated that spontaneous reaggregation is an effective approach for efficient viral transduction and genetic modification (8,9,20,21,38,39).

The microwell and hanging-drop reaggregation methods were developed to control the size and composition of pseudoislets. In the hanging-drop approach, islet cells are suspended in small liquid droplets (for example, distributed across inverted tissue-culture dish lids) and reaggregated into spheroid pseudoislets during culture (40). This approach can be laborious, but modified hanging-drop platforms that are compatible with automated cell seeding have facilitated larger-scale studies (41,42). Customized microwell plates also concentrate cells into small-diameter wells to achieve size-controlled clusters (10), and an inverse pyramidal microwell system validated in human pseudoislet studies is commercially available under the name of AggreWell (11,38). Each well can create thousands of pseudoislets with a single pipetting and centrifugation step, a platform efficient for larger-scale studies. In a direct comparison of these methods, Walker et al. (41) found that glucose-dependent insulin or glucagon

Table 1—Summary of established methods for human pseudoislet formation

Method	Approach	Advantages	Disadvantages	Key references
Spontaneous reaggregation	After dissociation, islet cells are introduced to ultra-low attachment plates and reaggregate spontaneously into pseudoislets	Accessible and widely validated in transduction experiments	Produces pseudoislets of heterogeneous size (although greater homogeneity can be achieved by reducing the volume of culture media and using gentle centrifugation)	Liu et al. (2019), Zaldumbide et al. (2013), Arda et al. (2016), Peiris et al. (2018), Bevacqua et al. (2021a & 2021b)
Microwell	After dissociation, islet cells are concentrated into small-diameter wells in customized microwell plates to achieve size-controlled clusters	Achieves pseudoislets of uniform size and efficient for large-scale experiments	Limited by cost of microwell plates	Liu et al. (2019), Hilderink et al. (2015), Yu et al. (2018)
Hanging drop	After dissociation, islet cells are suspended in small liquid droplets (for example, distributed across inverted tissue-culture dish lids), and gravity facilitates the formation of spherical clusters	Accessible and achieves pseudoislets of uniform size	Laborious (although modified hanging-drop platforms that are compatible with automated cell seeding have facilitated larger-scale studies)	Zuellig et al. (2017), van Krieken et al. (2019), Walker et al. (2020)

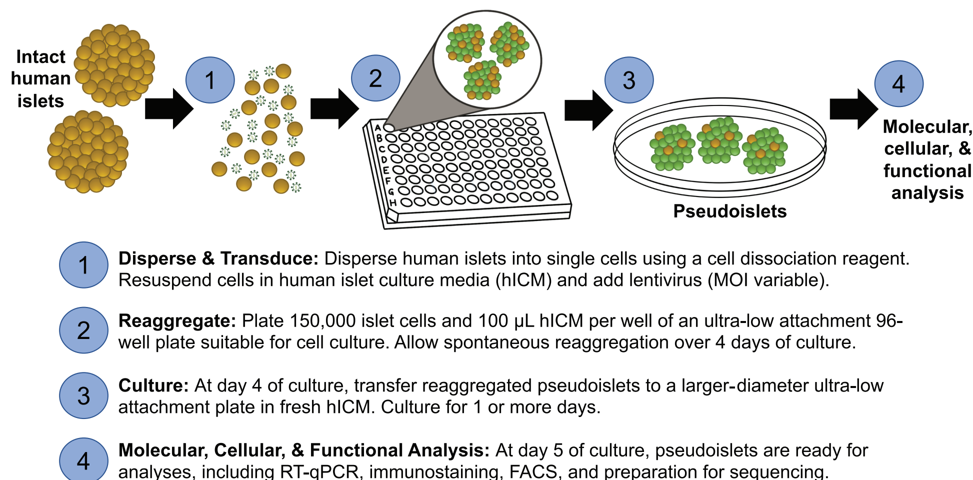

Figure 1—A visual protocol for pseudoislet formation with viral transduction for genetic modification studies. hICM, human islet culture media; MOI, multiplicity of infection; RT-qPCR, quantitative RT-PCR.

secretion was comparable in pseudoislets prepared with hanging drops or microwells. Thus, distinct reaggregation methods can produce pseudoislets with similar in vitro endocrine functions.

Each method for pseudoislet development in vitro has benefits and challenges. While microwells are efficient for larger-scale experiments (10,11), spontaneous or hanging-drop methods are more cost-effective for smaller-scale studies (38,40). Modified hanging-drop systems can be automated (41,42), but exposure to reagents with biohazardous potential (such as adenovirus or lentivirus for transduction experiments) is more easily contained within ultra-low attachment and microwell plates. Spontaneous reaggregation is widely validated in transduction experiments (8,9,21,38,39,43) and less costly, but this approach yields more heterogeneous pseudoislets. Finally, each laboratory may have empirical experiences that inform preferences in pseudoislet formation platforms: our group has found good formation of porcine and murine pseudoislets in microwell platforms, while human pseudoislet development is most effective with use of spontaneous aggregation. Each of these methods is compatible with subsequent molecular, cellular, and functional assays.

Pseudoislet Physiology and Function

The Importance of Islet and Pseudoislet Composition for Normal Function

For improvement of interpretation of most outcomes, the pseudoislet platform should ideally reconstitute important intrinsic and cell-cell mechanisms that modulate hormone secretion from islet cells. In humans, insulin-secreting β-cells, glucagon-secreting α-cells, and somatostatin-secreting δ-cells, respectively, constitute 50–75%, 15–30%, and 3–10% of the endocrine pancreas (2), though this composition appears to be age-dependent (44). Regulation of hormone secretion from islet cells is mediated by intrinsic, paracrine, and juxtacrine signaling, which is linked to islet architecture. For example, β-cell function is modulated by glucose metabolism (45) and electrical coupling between β-cells (37). In addition, intraislet signaling mediated by glucagon, somatostatin, and urocortin 3 modulates the "tone" of insulin output (46). Insulin secretion from β-cells is dysregulated upon dispersion, but reaggregation of islet cells restores GSIS (37,47). Moreover, these hallmark β-cell functions improve after birth and correlate with transcriptomic and epigenomic differences between juvenile and adult islets (21). Therefore, pseudoislet studies should ideally be adaptable for age-dependent islet studies. In our experience, pseudoislets made from juvenile donor islets retain properties of those donor islets, as assessed by transcriptomic and functional (GSIS) assessment (21,39). This is consistent with prior studies demonstrating that pseudoislets reconstitute native islet composition, cellular adhesions and connections, gene expression, hormone secretion, and developmental stage (11,21,39–41,48,49).

Cells and Gene Expression in Pseudoislets

Histological studies by us and others demonstrate that the ratio of input α-, β-, and δ-cells is preserved in cultured pseudoislets (39–41) (Fig. 2*A* and *B*). These findings are consistent with the observation that total insulin and glucagon content in pseudoislets and intact islets is similar (41,49). Additionally, after 5–6 days' hanging drop development or spontaneous reaggregation, pseudoislet α-, β-, and δ-cells are found intermingled, as they are in native islets (14,39,40).

Gene expression studies of pseudoislets also demonstrate the presence of the principal islet cell types and provide further understanding of pseudoislet signaling, function, and regulation. After 48 h of culture, pseudoislets and intact islets demonstrated similar transcript levels of genes involved in cell communication (*ITGB1*, *CDH1*, *LAMB1*, *GJA1*, *ITGB7*), secretory function (*INS*, *PCSK2*, *PDX1*, *GLP1R*, *PCSK1*), oxidative stress (*SOD1*,

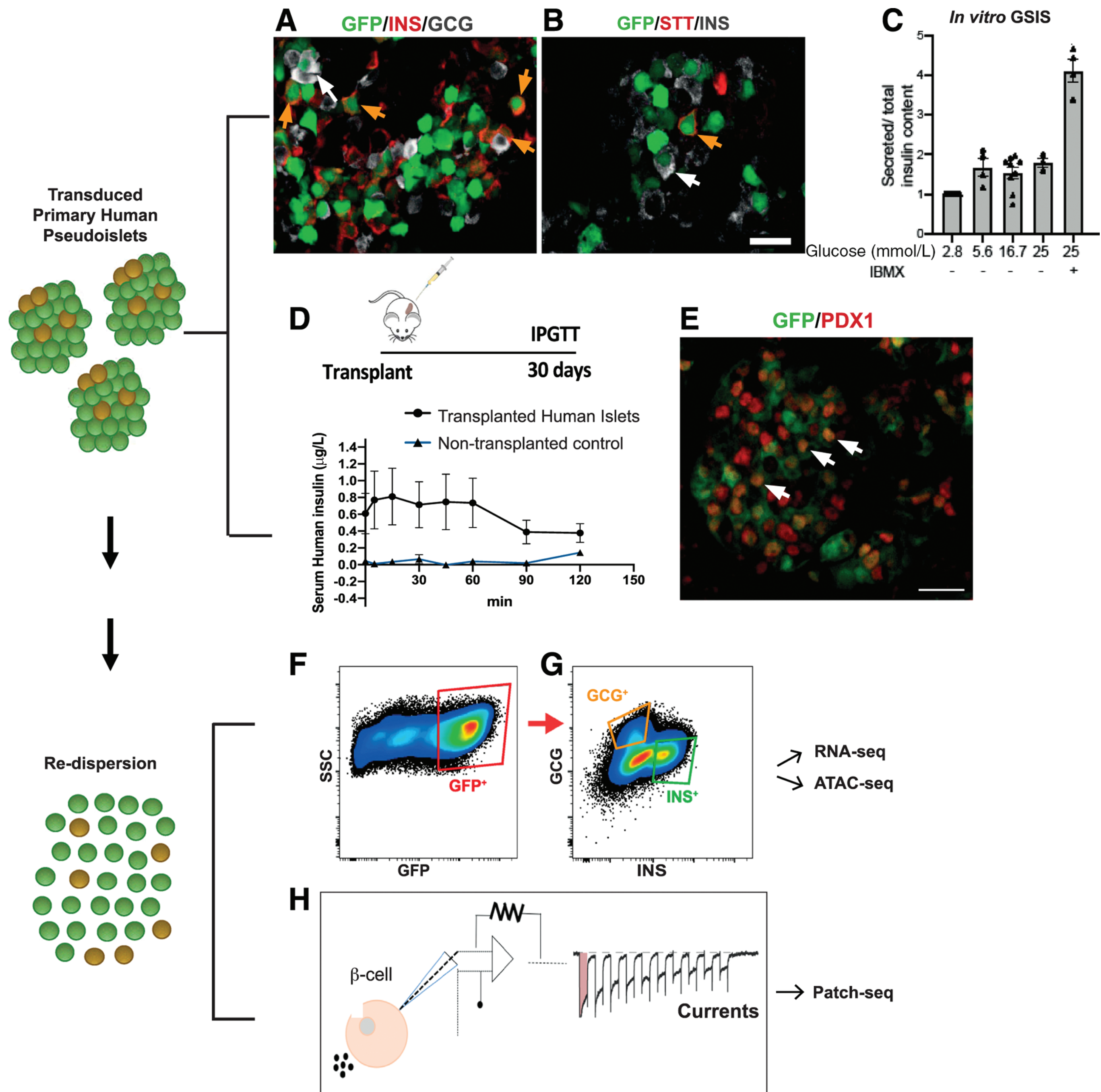

Figure 2—The human pseudoislet genetics platform allows assessment of islet-specific gene functionalities in vitro and in vivo. *A* and *B*: Dispersed human islet cells were transduced with lentiviruses coding for GFP under a constitutive promoter to show that all islet cell types can be efficiently transduced; immunostaining of human pseudoislets showing β (INS$^+$, orange arrows) (*A* and *B*), α (GCG$^+$, white arrows) (*A*), and δ (STT$^+$, orange arrows) (*B*) efficiently transduced (GFP$^+$) islet cells. Scale bar: 20 μm. *C*: Functionality of transduced pseudoislets can be assessed through GSIS in vitro. The data are normalized to 2.8 mmol/L glucose insulin level. *D*: Transduced pseudoislets (500/mouse) can be transplanted under the kidney capsule of immunocompromised mice and subjected to an intraperitoneal glucose tolerance test (IPGTT) in vivo, allowing assessment of longer-term effects of genetic manipulations. *E*: Grafts can be recovered and transduced cells (in this case, GFP$^+$ PDX$^+$ [white arrows]) further evaluated by immunostaining. Scale bar: 40 μm. *F* and *G*: Molecular analysis can also be performed following redispersion of pseudoislets transduced with lentiviruses coding for GFP under a constitutive promoter and FACS sorting of infected, GFP$^+$ (*F*) specific cell types (α: GCG$^+$; β: INS$^+$) (*G*). *H*: Following redispersion, single cells can also be used for patch-clamp or patch-seq. ATAC-seq, assay for transposase-accessible chromatin sequencing; RNA-seq, RNA sequencing.

SOD2, *CAT*), and apoptosis (*NFKB1*, *MAPK8*, *APAF1*, *MAPK10*, *NOS3*) by quantitative RT-PCR (11). In a subsequent bulk RNA-sequencing study, it was found that expression levels of factors involved in GSIS (including *GCK*, *SUR1*, *KCNJ11*, *CACNA1A*, and *GLP1R*) and β-cell identity (including *INS*, *MAFA*, and *NEUROD1*) were indistinguishable between islets and pseudoislets (49). Immunostaining confirmed pseudoislet expression of β-cell *PDX1* and

NKX6.1, α-cell *ARX*, and *MAFB*, *PAX6*, and *NKX2.2* in both β- and α-cells, like in native human islets (41).

Some studies report altered islet composition after pseudoislet formation. For example, insulin$^+$ cells can localize to the periphery of cultured pseudoislets (10–12,41), possibly reflecting a centrifugation step during reaggregation (10–12) or specific culture media (41). Additionally, bulk RNA-sequencing methods revealed that mRNAs encoding extracellular matrix (ECM), endothelial, exocrine, and ductal cell markers were reduced in pseudoislets compared with primary islets, indicating enrichment of endocrine populations and potential loss of nonendocrine components during dissociation and culture (49). In standard culture media, like CMRL-1066 or RPMI-1640 supplemented with serum, the reduction of ECM components after reaggregation of pseudoislets has been postulated to *facilitate* diffusion of nutrients and oxygen to endocrine cells in vitro or after transplantation (11). In addition, modulation of input cell types could enhance pseudoislet survival and function after transplantation (14,50). For example, in one study it was found that inclusion of mesenchymal stem cells improved survival and enhanced insulin secretion from pseudoislets (14). This effect was dependent on N-cadherin–mediated cell adhesion, reinforcing the importance of adhesion and cell-to-cell contact in pseudoislet function.

Islet Cell Hormone Secretion in Pseudoislets

Studies at "basal" glucose levels (2.8 mmol/L) show comparable insulin secretion in control intact islets and pseudoislets after 5 days of in vitro culture. However, with use of GSIS assays, some studies have observed that a step increase of glucose to 12 or 20 mmol/L evokes higher insulin secretion in size-controlled pseudoislets than in intact islets (40,49); the basis for these findings has not been determined. In our experience, static batch GSIS after genetic manipulation of pseudoislets, like lentiviral transduction, leads to a 1.5-fold to 2.0-fold increase of insulin output for up to 10 days of culture (8,39 and A. Urizar, Y. Hang, S.K.K., unpublished data) (Fig. 2*C*), and this output is enhanced by the addition of the potentiator 3-isobutyl-1-methylxanthine (IBMX), consistent with reports by others (10,37).

With use of dynamic perifusion, biphasic GSIS by pseudoislets has also been characterized (40,41,48). In most studies, there was no significant difference between intact human islets and human pseudoislets in first-phase, second-phase, or total insulin secretion after 6–7 days of culture (40,41), although one report observed a higher first-phase–to–second-phase GSIS ratio in pseudoislets relative to intact islets cultured for the same period (48). In addition to preservation of the biphasic insulin response to glucose, pseudoislets retain physiologic responses to the secretion potentiators glibenclamide (48), potassium (41), IBMX (8,21), palmitate, and forskolin (49). Pseudoislets and intact islets also have similar glucagon secretion, which is inhibited by high glucose and potentiated by IBMX, epinephrine, potassium, and L-arginine (8,41). Moreover, direct comparison of pseudoislets prepared via hanging drop or ultra-low attachment microwells reported very similar insulin and glucagon secretion at low and high glucose levels (41), demonstrating that distinct reaggregation methods yield similar function in pseudoislets.

Relatively longer-term culture of pseudoislets (>6 days in vitro) can enhance experimental assessment of drug exposure (51) or genetic modification (43). Batch GSIS, insulin content, and glucagon content remain stable in pseudoislets from 6 to 14 days of standard culture (49). In contrast to analyses of intact islets (52,53), three studies reported that pseudoislet function *improved* with extended culture (11,37,40). Furthermore, relatively smaller pseudoislets had enhanced GSIS in comparison with native intact islets in static batch assays up to 15 days under standard culture conditions (11). The functional advantage of smaller pseudoislets was attributed to greater resistance to hypoxia, as smaller diameter facilitates diffusion of nutrients to cells within the pseudoislet core (11). By a similar logic, it has been proposed that size-matched pseudoislets may tolerate hypoxia and long-term culture better than native islets because of the relative paucity of ECM encasing the multicellular structure (11). Thus, pseudoislet studies demonstrate how simple manipulation of pancreatic islets, such as dispersion and reaggregation, may be useful for understanding endocrine function and optimizing cell performance in clinical islet transplantation.

Transplantation Studies of Pseudoislet Function

Pseudoislet transplantation has been valuable for investigating durable in vivo properties, including after pseudoislet genetic manipulation (Fig. 2*D* and *E*). To date, the most common site for human pseudoislet transplantation studies is the renal subcapsular space of immunodeficient mice (8–15 [this is not an exhaustive reference listing]). In this setting, human pseudoislets and intact islets engrafted with equivalent efficiency, and pseudoislets performed at least as well as intact islets in rescuing hyperglycemia in streptozotocin-induced diabetic mice (11). Moreover, pseudoislet function was durable: glucose clearance after intraperitoneal glucose challenge 2 months after pseudoislet transplantation was comparable with that of untreated nondiabetic controls. In some cases, in vivo function of the transplanted pseudoislets was confirmed by reversion to diabetes after pseudoislet graft removal (11). Immunostaining of recovered grafts revealed that morphology, hormone expression, and cell localization of transplanted human pseudoislets resembled those of islets in situ (8,11,13,43). Additionally, pseudoislets appeared well revascularized in vivo, even potentially to a greater extent than intact islets. This observation has been attributed to the reduced or absent ECM capsule in

pseudoislets that otherwise might impede neovascularization (11,13).

Novel Functional and Molecular Approaches for Characterizing Pseudoislets

Whole-cell patch clamp can be used for assessment of electrophysiological function and hormone exocytosis from single cells recovered from pseudoislets (43) (Fig. 2*H*). Recent advances combining whole-cell patch-clamp measurements and single-cell RNA sequencing ("patch-seq") have allowed simultaneous assessment and correlation of quantitative physiologic measurements and gene expression in human islet cells (54). In combination with novel genetic pseudoislet approaches, patch-seq may illuminate genetic and functional pathways critical for pancreatic endocrine cell function.

Genetic Studies With Pseudoislet-Based Approaches

The application of genetics, especially loss-of-function approaches, to primary human islet cells has been an enduring challenge. Human stem cell–derived insulin-producing cells that resemble β-cells (47) and immortalized β-cell lines derived from human fetal pancreas like EndoC-βH3 (55,56) have provided surrogate systems for investigating human islet genetics. Despite the value of these models, they are fundamentally different from genuine pancreatic islet cells in terms of gene regulation, maturation, function, proliferation (57), and representation of β- and non-β-cell types, limiting their interpretation. Still, the quiescence and restricted duration of primary human islet cell in vitro culture impose restrictions on genetic studies that require efficient gene-delivery systems for transduction. Lentiviruses, retroviruses, adenoviruses, and adeno-associated viruses (AAVs) carrying cDNA-expressing constructs have been used for overexpression experiments in intact islets of mice and humans (58–62). However, overall cellular transduction in intact islets was low and inconsistent, ranging from 2% to 50%, reflecting the relative impermeability of the intact islet to viral transduction (58,59). Moreover, variable results from viral transduction of intact islets may arise from the viral type or subtype and the species origin of islets used (9,60).

Viral transduction studies showed that dispersion of murine islets improved transduction efficiency of pancreatic endocrine cells by retroviruses and lentiviruses (59). Interestingly, only lentiviruses resulted in efficient transduction of β-cells (25% transduced insulin$^+$ cells for lentiviruses, vs. <1% by retroviruses). However, islet cells were cultured as a dispersed monolayer following infection in this prior publication, precluding analysis in vivo. Multiple subsequent studies showed that viral transduction of pseudoislets could be applied for genetic studies of islets from rodents (20,42) and humans (8,9,21,39,41,43,48,63,64) (see Table 2). More than 70–80% of all islet cells can be transduced for gain- or loss-of-function studies (8,41,48,63) (see Fig. 2*A* and *B*). This approach can be adapted in multiple ways to achieve islet cell targeting, including use of *cis*-regulatory elements that promote specific transgene expression in β-cells (9,48) or α-cells (65). Likewise, this general

Table 2—Comprehensive list of studies with use of genetics in pseudoislets

Delivery	Genetic modification	Species	Reference(s)	Application
Lentiviruses	Gain of function	Human	Zaldumbide et al. (2013)	Misexpression of immune-evasion proteins
			Arda et al. (2016)	Misexpression of transcription factors
			Peiris et al. (2018)	Misexpression of transcription factors
			Bevacqua et al. (2021a)	Misexpression of transcription factors
		Rats	Caton et al. (2003)	Misexpression of various connexins
Lentiviruses	Loss of function (shRNAs)	Human	Peiris et al. (2018)	Silencing of transcription factor
			Harata et al. (2018)	Silencing of glucokinase
			Liu et al. (2019)	Technical report
			Liu et al. (2020)	Silencing of adipose triglyceride lipase
			Bevacqua et al. (2021a)	Silencing of transcription factor
Lentiviruses	Knockout (indels/deletions)	Human	Bevacqua et al. (2021b)	CRISPR/Cas9 knockout of transcription factors and noncoding regulatory DNA
Lentiviruses	Endogenous gene activation	Human	Bevacqua et al. (2021b)	CRISPR-A of a transcription factor and enhancer
Adenoviruses	Gain of function	Human	Walker et al. (2020)	Misexpression of biosensor (GCAMP), study of cell-cell interactions
			Furuyama et al. (2019)	Reprogramming of α-cells via ectopic expression of β-cell transcription factors
		Mice	van Krieken et al. (2019)	Amplification of the V1b receptor signaling
Herpes simplex virus	Gain of function	Human	Rabinovitch et al. (1999)	Misexpression of antiapoptotic gene (bcl-2)

method has been useful for studies of cadaveric human islets from a range of subjects, including neonates, children, adults, and subjects with diabetes, particularly when combined with cell-subtype transcriptome and chromatin analyses (8,21,39,43) (Fig. 2*F* and *G*).

For example, a study from our group (21) showed that misexpression of the transcription factor SIX3 in juvenile human islets enhanced GSIS. Normally, SIX3 expression in human islets is restricted to β-cells and not detected before age 10–11 years (21) or in rodent islets (4), suggesting that this transcription factor might play a key, species-specific role in functional maturation of β-cells, a possibility supported by recent studies with *SIX3* knockdown in human pseudoislets (39). In another study (8), we revealed—using gain- and loss-of-function studies in human pseudoislets—that the transcriptional repressor *BCL11A* had previously uncharacterized roles in regulating human β-cell function. Both SIX3 and BCL11A have been implicated by genome-wide association studies as candidate risk regulators in T2D (8,39,66,67). Thus, genetic studies in human pseudoislets have broken new ground in the long-standing drive to identify genetic regulators of human diabetes risk. Future application of pseudoislet genetics will benefit from methods to measure additional pseudoislet phenotypes (summarized in Fig. 2), including signaling pathways and cell-cell interactions that govern islet cell function (20,41,51).

Pseudoislet biology and genetic studies have also been useful for investigating islet transplantation outcomes (Fig. 2*D* and *E*). In one study, islet cells transduced with the *BCL2* gene were protected from cytokine-induced accumulation of toxic oxygen radicals and DNA fragmentation (63). Zaldumbide et al. (9) showed that human β-cells can be efficiently transduced with human cytomegalovirus-encoded US2 protein and serine proteinase inhibitor 9, and protected from autoreactive T cells, while retaining their hallmark functions. Lentiviral-mediated misexpression of the V1b receptor (a GPCR member of the vasopressin-oxytocin family) in mouse pseudoislets can improve function after engraftment into immunocompromised mice (42). These studies illuminate how pseudoislet studies might lead to translational applications in islet transplantation.

Conclusions and Outlook

Pseudoislets offer multiple experimental advantages, while maintaining crucial physiological functions of intact islets, features that can be exploited to address unsolved mysteries in human islet biology and diabetes. However, there are limitations that may be addressed in the future to optimize the use of this strategy. For example, cell loss during pseudoislet formation and the cost of human islet procurement could limit investigations requiring significant pseudoislet input. Continued optimization of islet isolation, islet culture, and pseudoislet formation methods will ameliorate these limitations. It will also be important to clarify how donor age or disease status impacts studies of pseudoislets, as most studies to date have focused on pseudoislets from previously healthy adult donors. Our group recently generated genetically modified pseudoislets from juvenile (21,39) and T2D human donors (R.J.B., S.K.K., unpublished data), but additional studies are needed to clarify the impact of age, disease status, and other clinical variables on possible molecular and functional differences of pseudoislet biology.

Pseudoislet biology offers many tantalizing possibilities for future studies—including the exciting possibilities of using nuclease-based site-specific gene editing on specific islet cell types and building chimeric pseudoislets to unveil signals that enhance islet cell differentiation (57). While space limits preclude extensive discussion, we foresee that pseudoislet approaches will be coupled with emerging approaches for high-throughput sequencing (55,68), specialized proteomics (69), islet electrophysiology (54), and studies of islets from large animal models with experimental advantages, including sheep and pigs (70–72). The pseudoislet organoid platform provides a strategy for incisive in vitro and in vivo studies of islet genetics, signaling, pharmacology, and integrated function. Pseudoislet studies could transform approaches for studying human islets in physiological and disease settings and promote relevant translational efforts for ameliorating or curing diabetes.

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