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Formation of Amyloid in Encapsulated Human Pancreatic and Human Stem Cell-Generated Beta Cell Implants

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Abbreviations

<i>BW</i>	<i>body weight</i>
<i>EPFL</i>	<i>Ecole Polytechnique Fédérale de Lausanne</i>
<i>hESC-PE</i>	<i>human embryonic stem cell-derived pancreatic endoderm</i>
<i>hiPSC-PE</i>	<i>human induced pluripotent stem cell-derived pancreatic endoderm</i>
<i>hu</i>	<i>human</i>
<i>IAPP</i>	<i>Islet Amyloid Polypeptide</i>
<i>IEQ</i>	<i>islet equivalents</i>
<i>Ins⁺</i>	<i>insulin-positive</i>
<i>IQR</i>	<i>interquartile range</i>
<i>NOD/SCID</i>	<i>nonobese diabetic/severe combined immune deficiency</i>
<i>NSG</i>	<i>nonobese diabetic/severe combined immune deficiency gamma, NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ</i>
<i>PT</i>	<i>post-transplant</i>
<i>SCID/BEIGE</i>	<i>severe combined immune deficiency, CBl7.Cg-Prkdc^{scid}Lyst^{bg-J}/CrI</i>
<i>ThioF⁺</i>	<i>Thioflavin S-positive</i>

Abstract

Detection of amyloid in intraportal islet implants of type 1 diabetes patients has been proposed as cause in their functional decline. The present study uses cultured adult human islets devoid of amyloid to examine conditions of its formation. After intraportal injection in patients, amyloid deposits <15µm diameter were identified in 5-12 percent of beta cell-containing aggregates, 3-76 months post-transplantation. Such deposits also formed in glucose-controlling islet implants in the kidney of diabetic mice but not in failing implants. Alginate-encapsulated islets formed amyloid during culture when functional, and in all intraperitoneal implants that corrected diabetes in mice, exhibiting larger sizes than in functioning non-encapsulated implants. After intraperitoneal injection in a patient, retrieved single capsules presented amyloid near living beta cells whereas no amyloid occurred in clustered capsules with dead cells. Amyloid was also demonstrated in functional human stem cell-generated beta cell implants in subcutaneous devices of mice. Deposits up to 35µm diameter were localized in beta cell-enriched regions and related to an elevated IAPP over insulin ratio in the newly generated beta cells. Amyloid in device-encapsulated human stem cell-generated beta cell implants marks formation of a functional beta cell mass but also an imbalance between its activated state and its microenvironment.

Introduction

Amyloid deposits are since more than a century considered as a characteristic of pancreatic islets in type 2 diabetes patients (1-3), but they also appear in type 1 diabetes patients (4) and in non-diabetic subjects above age 50 (5,6), although to a lesser extent. Several decades after their first description they were shown to consist of islet amyloid polypeptide (IAPP) (7), a secretory product of beta cells, that is normally co-secreted with insulin towards the blood circulation, but which can form aggregates under predisposing factors in the beta cells and their environment (8,9). Key elements in their formation are the amyloidogenic sequence in the human IAPP-molecule (10) and the causes that induce its local accumulation in islet tissue (11). The latter may result from a sustained elevated release under chronic hyperglycemia (8,12) and/or a dysfunctional state of the beta cells (13). It can also be caused by inadequate autophagy of beta cells leading to accumulation of IAPP oligomers and formation of fibrils which can be cytotoxic (14,15). Formation of extracellular amyloid deposits can alter interstitial flow and thus secondarily impair

beta cell functions. A study in type-2 diabetic baboons suggested that islet amyloid deposition predisposes to hyperglycemia (16). The question remains whether amyloid formation is an early process, and thus a causative factor in the development of type 2 diabetes or rather a consequence with further deterioration of the functional beta cell mass. This question is also relevant for implants in beta cell replacement therapies for type 1 diabetes recipients.

The occurrence of amyloid in human islet implants was first reported in immune-compromised mice receiving islets under the kidney capsule (17) and later in liver and in spleen (18). The deposits occupied a larger relative space when the islets had been micro-encapsulated before injection, which was attributed to their lack in vascularization (19). Detection of amyloid in intraportal islet implants in type 1 diabetes recipients (20,21) led to the view that it may be responsible, at least in part, for the progressive functional decline that is known to occur in the implants of these patients (22). Amyloid deposits have also been observed in subcutaneous implants of human islets embedded in an alginate layer that was placed in a device; they occurred with a mixture of debris near islets with a blunted secretory response after explantation; the patients exhibited only a marginal and transient implant function during the 3 to 6 month posttransplant period (23). It is unclear whether, and if so to which extent, amyloid can be formed by dying beta cells, a process that might be more pronounced in encapsulated implants which are subject to reduced oxygenation. It is also unknown whether use of islets from adult donors, some up to age 75 years (24) carries a higher risk for beta cell dysfunction in implants, in particular when exposed to their new microenvironment and the metabolic state of the recipient. These islet preparations may also contain amyloid deposits at start.

The present study used cultured adult human islet preparations in which no amyloid was detected to evaluate formation of deposits in their intraportal implants in type 1 diabetes patients. It examined the effect of encapsulation during culture as well as following transplantation in the peritoneal cavity of mice and of a patient. It investigated whether amyloid could also be formed by newly generated beta cells derived from human pancreatic progenitors.

Research Design and Methods

Human pancreatic islet cells following culture and transplantation

Human islet cells were prepared by our Beta Cell Bank which receives donor organs from Eurotransplant International Foundation (Leiden, The Netherlands) for its clinical transplant program. They were cultured in serum-free Ham's F-10 medium and analysed for cellular composition, in particular beta cell number and purity, and percent dead cells (25-27). In cultured islet preparations, dithizone staining is not an adequate marker for beta cell mass and its relationship with the plasma C-peptide levels achieved in C-peptide negative type 1 diabetes patients (27); grafts with minimally 2×10^6 beta cells/kg BW were consistently found to establish implants with plasma C-peptide >0.5 ng/ml at PT (post-transplant) month 2 (27); this corresponds to 4000 IEQ/kg BW (26). As in previous publications, the term "graft" is used for the preparation to be placed in the recipient, the term "implant" for the tissue formed after transplantation of the graft (26-29).

Clinical protocols and their outcome have been previously described (27-29). The present study was conducted on implants that were retrieved from six patients of these published series, each of whom had received 1, 2 or 3 intraportal injections of cultured islets at the listed beta cell number and purity (Table 1) leading to plasma human (hu-)C-peptide > 0.5 ng/ml post-transplantation. The 12 grafts were prepared from islet isolates (median donor age 46 years; IQR 37-55) after a median culture period of 5 days (IQR 3-7) and had a median insulin purity of 27% (IQR 17.5-38.0%) and median beta cell number of 2.2×10^6 beta cells/kg BW (IQR 1.4-2.8). Patient 3 received in addition an intraperitoneal graft of alginate-encapsulated islets, five years after the last intraportal injection when plasma C-peptide had been under 0.5 ng/ml for 9 months of continued maintenance immune therapy; this graft contained 2.4×10^6 beta cells/kg BW (29).

In vitro and in vivo laboratory studies were conducted with cultured islets that did not meet quantitative requirements for clinical grafts; the cells were used according to guidelines of Eurotransplant and approval by our institutional review committee (B.U.N 143201731194) and ethics committee for animal experimentation (following the European Community Council Directive 2010/63/EU). Preparations were examined for the presence of amyloid with or without alginate matrix. Islets at $15\text{-}20 \times 10^6$ cells/ml were suspended in 1.8% high guluronic acid alginate (Pronova UP LVG; Novamatrix, Oslo, Norway) before generating micro-droplets in a barium-calcium solution (29). Microcapsules were cultured in Ham's F10 medium (Lonza, Basel, Switzerland) with 6.1 or 20 mmol/L glucose, supplemented with L-glutamine, penicillin (10 000 U/mL), streptomycin (10 000 U/mL) and nicotinamide (0.5 mmol/L) for three weeks to examine

amyloid formation in vitro with cultured non-encapsulated islets as control (n=3). In vivo amyloid formation was assessed in implants that were available from a previously published study in which microcapsules had been injected in the peritoneal cavity of alloxan-diabetic NOD/SCID mice (7-to 8-wk-old males, Charles River, Saint-Germain-sur-l'Arbresle, France) and in which non-encapsulated islets had been implanted in the kidney capsule; hyperglycemia was corrected in 10/10 recipients of intraperitoneal capsules and in 4/12 of non-encapsulated islets (29).

Human stem cell-generated beta cell implants

The presence of amyloid was also examined in human stem cell-derived beta cell implants. This tissue was retrieved from devices that had been filled with human stem cell-derived pancreatic endoderm (SC-PE) before placement under the skin of immune-compromised mice. Samples originate from three published studies as indicated in Table 2: in the first, human embryonic (hE)SC-PE was loaded in TheraCyte™ devices and placed in NOD/SCID mice for retrieval at PT week 20 (30), in the second, hESC-PE was inserted in ViaCyte's Encaptra® devices and placed in NSG mice (Charles River) for explant at PT week 50 (31), in the third, human induced pluripotent (hiP)SC-PE had been injected in the device from Ecole Polytechnique Fédérale de Lausanne (EPFL-device) (32) and transplanted in SCID/BEIGE mice (Charles River) for retrieval at PT week 20 (33).

Histological and immunochemical analysis

Samples from cultured preparations were fixed in glutaraldehyde and embedded in araldite. Implants were fixed in 4% phosphate-buffered formalin (tissue from liver, kidney or devices) or in Bouin's solution (alginate capsules), and embedded in paraffin. After heat-induced antigen retrieval (2100-Retriever; Pickcell Laboratories, Lelystad, The Netherlands) in citrate buffer (Scytek Laboratories, Logan, UT, USA) staining was performed with guinea pig anti-insulin (1/2000; in house produced) and rabbit anti-IAPP (1/1000; Acris, Herford, Germany. Secondary antibodies were F(ab')₂ fragments of affinity-purified antibodies suited for multiple labelling, Alexa Fluor 647-conjugated donkey anti-guinea pig and Alexa Fluor Cy3-conjugated donkey anti-guinea pig (concentration 1/500 for both). IAPP-binding was visualised with immPRESS® HRP Horse Anti-Rabbit IgG Polymer Detection Kit, and counterstaining with hematoxylin. These fixation and processing methods had been found to allow detection of amyloid. The presence of

amyloid was shown by Thioflavin S staining (34) (Sigma T1892; Merck KgAa, Darmstadt, Germany), and confirmed by a Congo Red staining counterstained with Gill's hematoxylin. (as described (21); it was also visualized with HT60, Sigma-Aldrich, St. Louis, MO, USA) showing green birefringence under polarized light (no figure included) and examined for IAPP-positivity. Use of 5 μ m sections gave a lighter Congo Red staining than is usually observed with 10 μ m sections. Sections were analysed at both the Brussels and Uppsala laboratories to confirm presence and absence of amyloid.

Digital images were acquired on an Axioplan 2 (Carl Zeiss, Oberkochen, Germany) microscope with an Orca-R2 camera (Hamamatsu Photonics) and SmartCapture 3 software version 3.0.8 (DSUK, Cambridge, UK) or acquired with an Aperio CS2 (Leica Biosystems, Nußloch, Germany) and visualized with Pathomation software (Antwerp, Belgium). For automated segmentation analysis of the areas of Thioflavin S and DAPI (Sigma) positivity, large images were acquired with a BD Pathway 435 microscope and analysed in Attovision v1.6 (BD Biosciences, CA, USA). Data were processed using IPLab Software v4.02 (BD Biosciences, CA, USA). Descriptive statistics of relative Thioflavin S and insulin areas were obtained with Prism 8.4.2 (GraphPad Software, CA, USA).

Human stem cell-generated beta cells were assayed for their IAPP and insulin content. Cellular extracts were available from previously published studies (31,33). They had been prepared from retrieved implants that were gently dispersed using collagenase and washed before determining cell number and composition. Samples were then acid-extracted and stored. They were assayed for IAPP using an ELISA kit (EZHA 52-K, Merck, Darmstadt, Germany) (34) and for insulin using an in-house assay (25). Data were expressed per 10^3 beta cells, and compared with those obtained from cultured adult human islets. Statistical analysis (unpaired t-test with Welch's correction) was performed using Prism 8.4.2; statistical significance was assumed at $p < 0.05$.

Results

Cultured adult human islets devoid of amyloid form small deposits in intraportal implants in type 1 diabetes patients

Cultured adult human islets were devoid of amyloid as judged by absence of Thioflavin S-positive particles. In samples taken from the 12 grafts that were prepared for intraportal transplantation in six patients, only 1/1071 beta cell containing aggregates exhibited a positive inclusion (ThioF⁺, Table 1); its size was small relative to the insulin-positive (Ins⁺) surface area in that preparation (0.1%). Analysis of implants retrieved 3 to 76 months post-transplantation showed the presence of ThioF⁺ deposits in 47/737 beta cell containing aggregates (Table 1). In each of the six patients, aggregates of insulin-containing cells were identified in the liver and associated with an in vivo marker of functioning beta cells (plasma hu-C-peptide 0.1-2 ng/ml), the highest level being measured in the case with shortest PT follow-up, 3 months (Table 1). Amyloid was detected in five patients, however of small size (< 15 μm) and only occurring in 5 to 12% of the beta cell containing aggregates (Table 1, Fig 1 A-B). In the other case only two beta cell containing aggregates were detected which is insufficient to evaluate formation of amyloid.

Alginate-encapsulation of human islets induces amyloid formation in culture and in their intraperitoneal implants in mice and in a patient

When islets were micro-encapsulated in alginate and cultured for three weeks in basal (6 mM) or high (20 mM) glucose concentration, ThioF⁺ deposits were found in each condition, adjacent to living beta cells (Fig 2, A-B); they occupied an area of, respectively, 2.7 and 3.1% relative to that of insulin; no ThioF⁺ deposits were detected when encapsulated cells had been killed by hydrogen peroxide (H₂O₂, 0.5 mM, 120 min) at start of culture; non-encapsulated islets remained negative (data not shown).

When alginate-encapsulated human islets were peritoneally transplanted in diabetic NOD/SCID mice, instead of cultured, they corrected hyperglycemia in 10/10 recipients at plasma hu-C-peptide levels >2 ng/ml (29). Capsules retrieved at PT month 1-2 showed amyloid deposits in 4/4 normoglycemic mice, located near Ins⁺ cells and exhibiting a wide range in relative ThioF⁺ area over Ins⁺ area (median 10.2%; IQR 2.1-71.0). This was also the case in 5/5 normoglycemic mice examined at PT month 4-6 (median ThioF⁺ over Ins⁺ ratio 23.1; IQR 15.3-69.3) (Fig 2, C-D). Amyloid was also found in normalized recipients of non-encapsulated human islet implants under the kidney capsule (3/3) but not in mice with failing implants (8/8); deposits were smaller at PT month 1-2 (median ThioF⁺ over Ins⁺ ratio 4.5%; IQR 1.8-5.3) (Fig 2, E-F). Congo Red staining

confirmed the observations with Thioflavin S staining (Fig 2). All mice had received grafts with similar beta cell number (approximately 2×10^6 beta cells) (29).

Intraperitoneal injection of alginate-encapsulated islets in a type 1 diabetes patient resulted only for one week in C-peptide levels > 0.5 ng/ml (29). At PT week 12, a laparoscopy indicated that most capsules had clustered against the peritoneal wall and did not contain viable cells; a small number was single and floating and exhibited living cell aggregates with glucose-responsive beta cells (29). We now demonstrate absence of amyloid in the clustered capsules with debris and its presence in free-floating capsules with living beta cells (Fig 1, C-F).

Formation of amyloid in device-encapsulated human stem cell-generated beta cell implants in mice

Amyloid is also formed in encapsulated human stem cell-generated beta cells implants that have been placed in the subcutis of mice. We examined implants from three studies with human pluripotent stem cell-derived pancreatic endoderm (differentiation stage 4) as graft, the first two using hESC-PE with analysis at PT week 20 and 50, the third with hiPSC-PE and analysis at PT week 20 (30,31,33). Starting preparations contained $<10\%$ weakly Ins⁺ cells, most double-hormone positive, but no ThioF⁺ deposits (Table 2). The three studies also differed in the type of devices and mouse strains which adds to the variables that can influence processes within the device (Table 2). All implants were retrieved from mice with plasma hu-C-peptide levels >1 ng/ml, indicating establishment of a functional beta cell mass. ThioF⁺ deposits had been formed in each study group however at high variability in their relative surface area (ThioF⁺/DAPI) per section. They were preferentially localized near Ins⁺ cells as expressed by the 3 to 7-fold higher ratio of ThioF⁺area/Ins⁺ area (Table 2). Regions with a high proportion of insulin-positive cells ($>25\%$) exhibited a markedly higher ratio of ThioF⁺surface/Ins⁺ surface than those with a lower proportion ($<20\%$) (Table 2) but the low number of devices examined within the same experimental group interfered with statistical analysis. Localization of ThioF⁺ deposits coincided with positive Congo Red staining (Fig 3) and with IAPP-positivity (Fig 4). Ins⁺ cells co-stained for IAPP and are thus considered to be the source for measured IAPP in extracts of cell aggregates prepared from implants at PT week 50: 0.95 ± 0.50 ng IAPP for 13.0 ± 3.0 ng insulin per 10^3 beta cells (n=4). Comparison with the content in cultured adult human islets (0.06 ± 0.02 ng IAPP and

17.9 $\pm$ 1.6 ng insulin per 10³ beta cells, n=5) indicates a more than 15-fold higher IAPP content (p<0.04) for a 27% lower insulin content (p<0.04). Consequently, the molar ratio of cellular IAPP over insulin is significantly higher in stem cell-generated beta cells than in cultured adult human islets (10.7 $\pm$ 4.7 % versus 0.4 $\pm$ 0.2 %, p=0.02).

Discussion

Our clinical transplant program uses cultured human islet cell preparations that are characterized by cellular composition and hormone content (26,27). The present study shows that they are virtually devoid of amyloid which helps identify conditions for its formation after their implantation. The islet isolation procedure and subsequent culture may have discarded islet-associated amyloid deposits that are observed in pancreases of ageing subjects, occurring in around 1% of the islets in the age category of the donors for our preparations (5). Our data demonstrate that amyloid deposits were formed after intraportal transplantation in type 1 diabetes patients where they have been first described by Westermarck et al (20,21). They were found associated with insulin-positive cell clusters, up to more than 5 years post-transplantation, however only as small particles (<15 μ m diameter) in a minority of sectioned beta cell aggregates (6%). Their presence may mark a microenvironment where extracellular fibril formation was favoured following elevated release of IAPP and/or its intermediate by beta cells under sustained metabolic activation (8,12,35,36). This process may also be induced by intracellular fibrils in donor cells possibly causing cell death and leaving an extracellular nucleus for further accumulation (37). We assessed presence of extracellular deposits using classical light microscopical methods for their detection in tissue and did not perform electron microscopy for further analysis. Extracellular deposits may impair local circulation and lead to dysfunction and death of surrounding beta cells (15,38-41). Their low abundance raises the question whether they represent a major cause in the functional decline of intraportal implants. It can however not be excluded that affected beta cell clusters disappear together with the deposits (14), while non-affected become progressively involved in amyloid formation. Our short-term study (1-2 months post-transplantation) in diabetic mouse recipients of a cultured islet graft indicated that implants with failing beta cell function did not exhibit deposits, while these were found when hyperglycemia was corrected, be it of small size (<15 μ m diameter) and at low abundance.

Alginate-encapsulation of cultured human islets created a microenvironment that induced amyloid formation, supporting the view that reduced flow promotes accumulation of locally released IAPP (19). Formation did not occur during culture, but not when islet cells were killed shortly after encapsulation, indicating its dependence on living beta cells and excluding that deposits come from dying beta cells. It also occurred in vivo following intraperitoneal injection in diabetic mice. Quantitative retrieval of capsules 1 to 6 months after transplantation showed that each recipient contained capsules with amyloid, however with wide variation, among recipients, in its relative abundance to the beta cell mass. At PT month 1-2, median Thioflavin S-positive area over insulin-positive area was more than 2-fold higher than that in functional non-encapsulated implants in the kidney capsule, but this was not associated with a lower efficacy in correcting hyperglycemia (29). Our data do not exclude the possibility that amyloid formed in capsules progressively impaired neighbouring beta cells. This could be investigated by longitudinal in vivo analysis of recipients prior to retrieval of capsules with ThioF⁺ over Ins⁺ ratio around 90% as occurred at PT month 1 as well as at PT month 5. The question can also be addressed by separating capsules with and without amyloid and comparing their respective beta cell number and function.

We also observed amyloid formation in alginate-encapsulated human islets containing glucose-responsive beta cells that had been retrieved as suspended single capsules from a type 1 diabetes recipient of an intraperitoneal implant (29). On the other hand no amyloid and no living cells were detected in capsules that had clustered against the peritoneal wall, suggesting that cell death occurred rapidly after implantation. It is however unknown how long amyloid that is formed within capsules remains visible after cell death, and similarly for deposits should these be present in the islet preparation before encapsulation. The clearance mechanisms for amyloid are not yet fully understood (42) and may differ in the microenvironment of a capsule. Better insights will help interpret the significance of amyloid in encapsulated implants that contain both living and dead cells such as in alginate-embedded human islets inserted in a macro-encapsulation device before subcutaneous implantation in type 1 diabetes patients (23).

Our study on encapsulated implants shows that amyloid can not only be formed by beta cells isolated from adult human donor organs but also by beta cells that are newly generated in situ by human pluripotent stem cell-derived pancreatic progenitors. It was detected in subcutaneous devices that had been filled with hESC-or hiPSC-derived pancreatic endoderm and examined 5 and 12 months after transplantation for presence of Thioflavin S-, Congo Red- and IAPP-positive

deposits. These were localized near clusters of newly formed beta cells and occupied a surface area that was highest in regions with high percentages of insulin-positive cells. These regions are responsible for the insulin-producing capacity of the implants. Previous work has shown that the hormonal and metabolic function of stem cell-generated beta cell implants depends on the number of formed beta cells and correlates with their relative proportion (31,33). Our observations further support the view that amyloid deposits in beta cell implants represent a marker for regions with high beta cell secretory activity in a microenvironment with reduced diffusion. The variability in beta cell formation among animals within the same experimental group (31,33) and the differences in beta cell density within the same device limit further conclusions from the present study that has been conducted on few devices. It is thus uncertain whether the differences in data collected from the various experimental conditions express influences by the used cell preparations and their genetic origin, by the type of devices, and/or by the degree and duration of elevated beta cell function in vivo. Combined measurement of plasma hu-C-peptide and IAPP under basal and elevated glycemia could have indicated whether a disproportionate release of IAPP had occurred for implants with high amyloid density; the limited number of examined devices and shortage in plasma samples and volume did not allow us to conduct this analysis. We did measure both peptides in extracts of cell aggregates that had been prepared from implants retrieved at post-transplant month 12. When expressed per 10^3 beta cells, cellular insulin content was 27% lower than that in cultured adult human islets but the corresponding IAPP content was 15-fold higher which led to an IAPP over insulin ratio of 10.7%, which is much higher than the ratios in extracts of human islets (0.4% in the present study, 0.7% and 1.8% in previous studies (12,43) and in plasma of non-diabetic patients (around 1%) (44). Perfusion of cells retrieved from implants can indicate whether their IAPP release is also high in absolute and/or relative terms and thus reflect a characteristic of beta cells under sustained metabolic activation. These higher IAPP levels can then induce amyloid formation in sites with slow diffusion as expected to occur in a device.

It can be concluded that device-encapsulated human stem cell-generated beta cell implants in immune-compromised rodents represent a new model for studying the sequence of events in formation of amyloid as well as for further defining its significance as marker for the functional state of its neighbouring beta cells and their microenvironment.

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Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Figure Legends

Figure 1. Amyloid in human islet implants retrieved from type 1 diabetes patients who received a non-encapsulated graft in the liver (A, B-representative sections from recipient 6) or an alginate-encapsulated graft in the peritoneal cavity (C-F). Intrahepatic implants exhibited small Congo Red- (A) and Thioflavin S-positive deposits (green, B) near insulin-positive cells. Intraperitoneal implants were retrieved as clustered capsules embedded in fibrotic tissue (C, D- hematoxylin eosin) or as floating single capsules (E, F). Clustered capsules did not contain cells or Congo Red-positive material (C). Single capsules presented Congo Red-(E) and Thioflavin S-positive deposits (green, F) near insulin-positive cells (red, DAPI white, F). Scale bar = 50 μ m.

Figure 2. Amyloid in alginate-encapsulated human islets following culture (A, B-representative for n=3 at 20 mM glucose for three weeks) or transplantation in the peritoneal cavity of NOD/SCID mice (C, D- representative for capsules that can be found in functioning implants at PT month 1 and 6) with comparison to non-encapsulated implants in the kidney capsule (E, F- representative for functioning implants at PT month 1-2, n=3). Thioflavin S-fluorescence (green) shows amyloid deposits within capsules (A, C), that also contain insulin-positive cells (Insulin red, DAPI white). Congo Red-staining confirms presence of amyloid in these conditions (B, D). Thioflavin S- and Congo Red-positive deposits in non-encapsulated implants are smaller and less abundant (E, F). Scale bar = 50 μ m.

Figure 3. Amyloid in device-encapsulated human stem cell-generated beta cell implants placed in the subcutis of immune-compromised mice. At PT week 50, implants of hESC-PE in Encaptra® device were found to exhibit Thioflavin S- (green, A) and Congo Red (B)-positive deposits near insulin-positive cells. This was also the case in hiPSC-PE implants in an EPFL-device at PT week 20, whereby the same device exhibited regions with high amyloid density and high density in insulin-positive cells (C, D) and regions with almost no amyloid positivity and low density in insulin-positive cells (E, F) (C and E: Thioflavin S green, Insulin red, DAPI white; D and F Congo Red). Scale bar = 50 μ m.

Figure 4. IAPP-staining of amyloid-containing region in device-encapsulated hiPSC-PE-generated beta cell implant at PT week 20. Consecutive sections show IAPP-positivity in insulin-positive cells (dark brown stain in B coinciding with the red cellular insulin-positivity in A) as well as in amyloid deposits (light brown in B) that are Thioflavin S-positive (green, A) and Congo Red-positive (C). Scale bar = 50 μ m.

References

1. Opie EL. The relation of diabetes mellitus to lesions of the pancreas. Hyaline degeneration of the islands of Langerhans. *J Exp Med.* 1901;25(5):527-540.

2. Höppener J, Ahrén B, Lips C. Islet amyloid and type 2 diabetes mellitus. *N Engl J Med*. 2000;343(6):411-419.
3. Warren S, Le Compte P, Legg M. Qualitative pancreatic changes in diabetes mellitus. In: *The pathology of diabetes mellitus*. Philadelphia: Lea & Febiger; 1966:53-89.
4. Westermark GT, Krogvold L, Dahl-Jorgensen K, et al. Islet amyloid in recent-onset type 1 diabetes-the DiViD study. *Ups J Med Sci*. 2017;122(3):201-203.
5. Bell ET. Hyalinization of the islets of Langerhans in nondiabetic individuals. *Am J Pathol*. 1959;35(4):801-805.
6. Westermark P. Quantitative studies of amyloid in the islets of Langerhans. *Ups J Med Sci*. 1972;77(2):91-94.
7. Westermark P, Wernstedt C, Wilander E, et al. A novel peptide in the calcitonin gene related peptide family as an amyloid fibril protein in the endocrine pancreas. *Biochem Biophys Res Commun*. 1986;140(3):827-831.
8. Montane J, Klimek-Abercrombie A, Potter KJ, et al. Metabolic stress , IAPP and islet amyloid. *Diabetes Obes Metab*. 2012;14(Suppl 3):68-77.
9. Potter KJ, Werner I, Denroche HC, et al. Amyloid formation in human islets is enhanced by heparin and inhibited by heparinase. *Am J Transplant*. 2015;15(6):1519-1530.
10. Moriarty DF, Raleigh DP. Effects of sequential proline substitutions on amyloid formation by human amylin₂₀₋₂₉. *Biochemistry*. 1999;38(6):1811-1818.
11. Johnson K, O'Brien T, Betsholtz C, et al. Islet amyloid, islet-amyloid polypeptide, and diabetes mellitus. *N Engl J Med*. 1989;321(8):513-518.
12. Hou X, Ling Z, Quartier E, et al. Prolonged exposure of pancreatic beta cells to raised glucose concentrations results in increased cellular content of islet amyloid polypeptide precursors. *Diabetologia*. 1999;42(2):188-194.
13. Huang C, Lin C, Haataja L, et al. High expression rates of human islet amyloid polypeptide induce endoplasmic reticulum stress-mediated β -cell apoptosis, a characteristic of humans with type 2 but not type 1 diabetes. *Diabetes*. 2007;56(8):2016-2027.

14. De Koning EJP, Van Den Brand JJG, Mott VL, et al. Macrophages and pancreatic islet amyloidosis. *Amyloid*. 1998;5(4):247-254.
15. Jurgens CA, Toukatly MN, Fligner CL, et al. β -cell loss and β -cell apoptosis in human type 2 diabetes are related to islet amyloid deposition. *AJPA*. 2011;178(6):2632-2640.
16. Guardado-Mendoza R, Davalli AM, Chavez AO, et al. Pancreatic islet amyloidosis, β -cell apoptosis, and α -cell proliferation are determinants of islet remodeling in type-2 diabetic baboons. *Proc Natl Acad Sci USA*. 2009;106(33):13992-13997.
17. Westermark P, Eizirik DL, Pipeleers DG, et al. Rapid deposition of amyloid in human islets transplanted into nude mice. *Diabetologia*. 1995;38(5):543-549.
18. Westermark GT, Westermark P, Nordin A, et al. Formation of amyloid in human pancreatic islets transplanted to the liver and spleen of nude mice. *Ups J Med Sci*. 2003;108(3):193-203.
19. Bohman S, Westermark GT. Extensive amyloid formation in transplanted microencapsulated mouse and human islets. *Amyloid*. 2012;19(2):87-93.
20. Westermark G, Westermark P, Berne C, et al. Widespread amyloid deposition in transplanted human pancreatic islets. *N Engl J Med*. 2008;359(9):977-979.
21. Westermark GT, Davalli AM, Secchi A, et al. Further evidence for amyloid deposition in clinical pancreatic islet grafts. *Transplantation*. 2012;93(2):219-223.
22. Shapiro MJ, Ricordi C, Hering BJ, et al. International trial of the Edmonton protocol for islet transplantation. *N Engl J Med*. 2006;355(13):1318-1330.
23. Carlsson P, Espes D, Sedigh A, et al. Transplantation of Macro-encapsulated Human Islets within the Bioartificial Pancreas ansplantation of Macro-encapsulated Human Islets within the Bioartificial Pancreas β Air to Patients with Type 1 Diabetes Mellitus. *Am J Transplant*. 2018;18(7):1735-1744.
24. Nano R, Kerr-conte JA, Scholz H, et al. Heterogeneity of Human Pancreatic Islet Isolation Around Europe : Results of a Survey Study. *Transplantation*. 2020;104(1):190-196.

25. Ling Z, Pipeleers DG. Prolonged exposure of human β cells to elevated glucose levels results in sustained cellular activation leading to a loss of glucose regulation. *J Clin Invest*. 1996;98(12):2805-2812.
26. Keymeulen B, Ling Z, Gorus F, et al. Implantation of standardized beta-cell grafts in a liver segment of IDDM patients: graft and recipients characteristics in two cases of insulin-independence under maintenance immunosuppression for prior kidney graft. *Diabetologia*. 1998;41(4):452-9.
27. Keymeulen B, Gillard P, Mathieu C, et al. Correlation between beta cell mass and glycemic control in type 1 diabetic recipients of islet cell graft. *Proc Natl Acad Sci USA*. 2006;103(46):17444-17449.
28. Gillard P, Hilbrands R, Van De Velde U, et al. Minimal functional β -cell mass in intraportal implants that reduces glycemic variability in type 1 diabetic recipients. *Diabetes Care*. 2013;36(11):3483-3488.
29. Jacobs-Tulleneers-Thevissen D, Chintinne M, Ling Z, et al. Sustained function of alginate-encapsulated human islet cell implants in the peritoneal cavity of mice leading to a pilot study in a type 1 diabetic patient. *Diabetologia*. 2013;56(7):1605-1614.
30. Motté E, Szepessy E, Suenens K, et al. Composition and function of macroencapsulated human embryonic stem cell-derived implants: comparison with clinical human islet cell grafts. *Am J Physiol Endocrinol Metab*. 2014;307(9):E838-46.
31. Robert T, De Mesmaeker I, Suenens KG, et al. Functional beta cell mass from device-encapsulated hESC-derived pancreatic endoderm achieving metabolic control. *Stem Cell Reports*. 2018;10(3):739-750.
32. Lathuilière A, Cosson S, Lutolf MP, et al. A high-capacity cell macroencapsulation system supporting the long-term survival of genetically engineered allogeneic cells. *Biomaterials*. 2014;35(2):779-791.
33. Robert T, De Mesmaeker I, Van Hulle F, et al. Cell mass increase associated with formation of glucose-controlling β -cell mass in device- encapsulated implants of hiPS-derived pancreatic endoderm. *Stem Cells Transl Med*. 2019;8(12):1296-1305.

34. Marzban L, Trigo-gonzalez G, Verchere CB. Processing of Pro-Islet Amyloid Polypeptide in the Constitutive and Regulated Secretory Pathways of β cells. *Mol Endocrinol*. 2005;19(8):2154-2163.
35. Courtade JA, Klimek-Abercrombie AM, Chen Y-C, et al. Measurement of pro-islet amyloid polypeptide (1-48) in diabetes and islet transplants. *J Clin Endocrinol Metab*. 2017;102(7):2595-2603.
36. Pipeleers D, Chintinne M, Denys B, et al. Restoring a functional β -cell mass in diabetes. *Diabetes, Obes Metab*. 2008;10(Suppl. 4):54-62.
37. Westermark P, Andersson A, Westermark GT. Islet amyloid polypeptide, islet amyloid and diabetes mellitus. *Physiol Rev*. 2011;91(3):795-826.
38. Mukherjee A, Scheihing DM, Salvadores N, et al. Induction of IAPP amyloid deposition and associated diabetic abnormalities by a prion-like mechanism. *J Exp Med*. 2017;2014(9):2591-2610.
39. Potter KJ, Abedini A, Marek P, et al. Islet amyloid deposition limits the viability of human islet grafts but not porcine islet grafts. *Proc Natl Acad Sci USA*. 2010;107(9):4305-4310.
40. Verchere CB, D'Alessio DA, Palmiter RD, et al. Islet amyloid formation associated with hyperglycemia in transgenic mice with pancreatic beta cell expression of human islet amyloid polypeptide. *Proc Natl Acad Sci USA*. 1996;93:3492-3496.
41. Paulsson JF, Westermark GT. Aberrant processing of human proislet amyloid polypeptide results in increased amyloid formation. *Diabetes*. 2005;54:2117-2125.
42. Gupta D, Leahy JL. Islet amyloid and type 2 diabetes : overproduction or inadequate clearance and detoxification ? *J Clin Invest*. 2014;124(8):3292-3294.
43. Gasa R, Gomis R, Casamitjana R, Novials A. High glucose concentration favors the selective secretion of islet amyloid polypeptide through a constitutive secretory pathway in human pancreatic islets. *Pancreas*. 2001;22(3):307-310.
44. Hull RL, Westermark GT, Westermark P, Kahn SE. Islet amyloid: a critical entity in the pathogenesis of type 2 diabetes. *J Clin Endocrinol Metab*. 2004;89(8):3629-3643.

Table 1. Characteristics of type 1 diabetes recipients of intraportal islet cell implant that was examined for amyloid

Recipient	1	2	3	4	5	6
Sex	Male	Male	Female	Female	Male	Male
Age (y)	54	40	61	61	56	62
Immune suppression						
- Induction	Cortic	ATG -Cortic	ATG-Cortic	ATG-Cortic	ATG-Cortic	ATG-Cortic
- Maintenance	Cy-Aza-Cortic	Tacr-MMF	Tacr-MMF	Tacr-MMF	Tacr-MMF	Tacr-MMF
Number of intraportal grafts	1	2	2	2	3	2
Beta cells per graft:						
- Number (10^6 /kg BW)	0.9	2.4-2.2	3.0-2.8	4.2-1.4	2.3-1.6-0.8	2.8-2.0
- Purity (% of total cells)	52	35-44	22-19	27-26	27-6-14	17-39
Beta cell function of implants						
Last value before retrieval:						
- hu-C-peptide (ng/ml)	0.1	0.3	0.4	2.0	0.9	0.5
- clamp (% controls)	ND	5	5	ND	19	13
Implant retrieval						
PT month	20	55	76	3	31	65
Autopsy	Bronchus carc.			Brain hemor.	Pancreas carc	Hypoglyc. Coma
Biopsy		Cholecyst.	Laparoscopy			

Number of beta cell aggr with ThioF ⁺ /total beta cell aggr						
- in graft (start)	0/225	0/55-0/67	1/56-0/52	0/53-0/68	0/53-0/78-0/68	0/162-0/134
- in implant after retrieval	6/74	1/15	0/2	23/491	1/21	16/134

ATG: antithymocyte globulin; Aza: azathioprine; Cortic: corticoids; Cy: cyclosporin; MMF: mycophenolate mofetil; Tacr: tacrolimus;

Carc: carcinoma; Cholecyst: cholecystectomy; Hemor: hemorrhage; Hypoglyc: hypoglycaemic;

Aggr: aggregates; ND: not determined; PT: post-transplant; ThioF⁺: thioflavin-positive inclusion.

Table 2. Presence and Relative Abundance of Amyloid in Device-Encapsulated Human Stem Cell-Generated Implants in Subcutis of Mice

Condition (n)				Mouse strain	Device	Ins+area/DAPI (%)	ThioF ⁺ area/DAPI (%)	ThioF ⁺ area/Ins+ area (%)
hESC-PE	Graft	pretransplant	(2)			5.3 (4.6-6.0)	0	0
	Implant	PT wk 20	(4)	NOD/SCID	TheraCyte™	12.0 (9.6-21.7)	0.1 (0-0.2)	0.7 (0-1.5)
		PT wk 50	(3)	NSG	Encaptra®	12.6 (7.8-24.0)	0.4 (0.2-3.9)	4.5 (1.5-16.3)
hiPSC-PE	Graft	pretransplant	(3)			4.7 (1.0-7.8)	0	0
	Implant	PT wk 20	(2)	SCID/BEIGE	EPFL device	22.3 (16.9-27.7)	6.2 (0.1-12.2)	22.5 (0.9- 44.1)
		<i>-region with > 25% Ins+cells</i>				32.2 (26.8 -37.5)	12.7 (0.4-25.1)	34.1(1.4-66.8)
		<i>-region with < 20% Ins+cells</i>				14.7 (10.4-18.9)	0.4 (0-0.7)	1.9 (0- 3.7)
Data represent median with IQR, determined in (n) graft preparations and implants, each analysed on 1074 to 14229 DAPI-positive cells.

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Thioflavin S, Congo Red and IAPP Staining
in Device-Encapsulated Human Stem Cell-Generated Beta Cell Implant

hiPSC-PE at PT wk 20
Region High in Ins⁺ cells

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