Review

MOLECULAR METABOLISM

Intraocular *in vivo* imaging of pancreatic islet cell physiology/pathology

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ABSTRACT

Background: Diabetes mellitus has reached epidemic proportions and requires new strategies for treatment. Unfortunately, the efficacy of treatment regimens on maintaining/re-gaining functional beta cell mass can, at the present, only be determined indirectly. Direct monitoring of beta cell mass is complicated by the anatomy of the endocrine pancreas, which consists of thousands to a million of discrete micro-organs, i.e. islets of Langerhans, which are scattered throughout the pancreas.

Scope of review: Here, we review the progress made over the last years using the anterior chamber of the eye as a transplantation site for functional imaging of pancreatic islet cells in the living organism. Islets engrafted on the iris are vascularized and innervated and the cornea, serving as a natural body-window, allows for microscopic, non-invasive, longitudinal evaluation of islet/beta cell function and survival with single-cell resolution in health and disease.

Major conclusions: Data provided by us and others demonstrate the high versatility of this imaging platform. The use of 'reporter islets' engrafted in the eye, reporting on the status of *in situ* endogenous islets in the pancreas of the same animal, allows the identification of key-events in the development and progression of diabetes. This will not only serve as a versatile research tool but will also lay the foundation for a personalized medicine approach and will serve as a screening platform for new drugs and/or treatment protocols. 'Metabolic' islet transplantation, in which islets engrafted in the eye replace the endogenous beta cells, will allow for the establishment of islet-specific transgenic models and 'humanized' mouse models as well as serving as the basis for a new clinical transplantation site for the cure of diabetes.

Keywords Diabetes mellitus; Type 1 diabetes; Type 2 diabetes; Pancreatic islet; Pancreatic beta cell; Live-cell imaging; Fluorescence microscopy; *In vivo* imaging; Anterior chamber of the eye

1. INTRODUCTION

Diabetes mellitus has reached epidemic proportions with increasing numbers of patients with both Type 1 (T1DM) and Type 2 diabetes (T2DM). In addition to the 415 million adults who are estimated to currently have diabetes, there are 318 million adults with impaired glucose tolerance, which puts them at high risk of developing the disease in the future and will contribute to the 642 million diabetes patients expected by 2040 [1]. Moreover, the International Diabetes Federation estimates that currently 193 million people suffering from the disease are undiagnosed and, consequently, at risk of developing diabetes complications.

T1DM is caused by an autoimmune destruction of insulin-producing pancreatic beta cells. However, the presence of circulating C-peptide in T1DM patients with a long history of the disease [2] suggests that some beta cells either escape the immune attack or that new beta cells are generated.

The majority of diabetic patients suffer from T2DM, a condition that develops when pancreatic beta cells fail to provide the organism with sufficient amounts of insulin to keep blood glucose levels within physiological limits. The relative insulin deficiency in T2DM is caused by beta cell failure to produce/secrete sufficient amounts of insulin to overcome peripheral insulin resistance mainly in skeletal muscle, fat, and liver.

Consequently, for both T1DM and T2DM, treatment strategies have to be developed that aim at protecting, preserving, or re-establishing functional beta cell mass. Unfortunately, the efficacy of treatment regimens on maintaining/re-gaining functional beta cell mass at present can only be determined indirectly, i.e. by measuring blood glucose levels in combination with circulating insulin/C-peptide levels. Direct monitoring of beta cell mass is complicated by the anatomy of the endocrine pancreas, which consists in humans of approximately 1 million discrete micro-organs, i.e. islets of Langerhans, scattered throughout the pancreas. In addition, beta cell mass can be highly variable between healthy individuals, making it impracticable to use an absolute number as a diagnostic tool. On the other hand, non-invasive, long-term monitoring of functional beta cell mass within the same individual will be an important step in the direction of a personalized medicine approach to combat this disease. A recent review describes in detail the current state in the generation of imaging probes and

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respective imaging modalities, e.g. magnetic resonance imaging (MRI), positron emission tomography/single-photon emission computed tomography (PET/SPECT), and bioluminescence imaging [3]. While these techniques will eventually allow quantification of beta cell mass *in vivo*, they will not enable quantification of subtle changes at the sub-islet level due to lack of spatial resolution. Such changes include changes in intra-islet blood flow, innervation, and beta cell heterogeneity in function and survival. As optical imaging techniques including confocal microscopy and optical coherence tomography to monitor pancreatic islet function have been discussed [4,5], here, imaging was often combined with either the exteriorized pancreas approach or with implantation of an optical body-window. The latter allows monitoring pancreatic islet function only for a few weeks.

In 2008, we presented an imaging approach that allows for monitoring pancreatic islet function and survival non-invasively, longitudinally at single-cell resolution in the living animal [6,7]. We transplanted isolated islets into the anterior chamber of the eye (ACE), where they engraft on the iris and become innervated and vascularized. By using the cornea as a natural body-window, these islets are readily available for functional microscopic imaging.

The ACE has been a transplantation site for a long time. van Dooremaal first described introducing/implanting different types of objects (e.g. pieces of paper) and tissues (e.g. pieces of skin) into the ACE of dogs and rabbits in 1873 [8]. Since then, the ACE has been used as a transplantation site for a variety of tissues ranging from ovaries [9], prostatic tissue [10], peripheral and central nervous tissues [11–13], kidney glomeruli [14], and pancreatic tissue [15–18]. With this information at hand, we hypothesized that the ACE should represent a unique transplantation site for pancreatic islets utilizing the cornea as a natural body-window for functional *in vivo* imaging of islet cells.

In the present review, we discuss the progress that has been made over the last years using this approach to study pancreatic islet/beta cell function and survival in health and disease. A schematic illustration summarizing the use of the ACE-based *in vivo* imaging technique is given in Figure 1.

2. PANCREATIC ISLET TRANSPLANTATION TO THE ACE

2.1. The procedure

The procedure of islet transplantation into the ACE of mice is described in great detail in a step-by-step protocol [7] as well as in a video tutorial [19]. In brief, pancreatic islets are isolated by standard procedures by either collagenase digestion or micro-dissection and aspirated into a blunt 27-G cannula in a minimal volume (10–20 μl). The anesthetized recipient-mouse is placed under a stereomicroscope, and the cornea is punctuated with the cannula between apex and limbus of the cornea. After gently inserting the cannula into the ACE, the islets are placed onto the iris by slow injection. The above-mentioned papers give a detailed layout of the experimental design including choice of donor and recipient mice, choice of anesthetics, as well as a description of used materials and equipment. Full engraftment of islets takes place within four weeks after transplantation. Engrafted islets can be monitored by various microscopic techniques ranging from simple light microscopy, fluorescence microscopy to high-resolution laser-scanning confocal microscopy/two-photon laser scanning microscopy.

In general, a syngeneic transplantation strategy is preferred; however, the broad range of potential donor mice with desired genetic modifications and a different genetic background requires an allogeneic approach and thus the use of immune-deficient recipient mice (e.g. nude mice, Rag1$^{-/-}$ mice, or NSG mice) to avoid immunological responses. Similarly, using immune-deficient mice as recipients is required when performing xenotransplantation to generate a 'humanized' mouse model (see section 2.4.2.). Given that the eye is an immune-privileged site, the need for immune-deficient mice might seem counterintuitive. However, it should be stressed that during the process of islet vascularization in the ACE, immune-privilege is broken. This observation allows for studying immune processes involved in islet graft rejection or mechanisms involved in T1DM development (see section 3.). The fact that the ACE can also be used for immunological studies is of particular importance when trying to identify novel and efficient strategies for intervention.

2.2. Islet vascularization in the ACE

In the pancreas, pancreatic islets are interspersed by a dense network of capillaries that guarantees the efficient exchange of oxygen, nutrients, and hormones between endocrine cells and the blood circulation of the body. Vascularization of the islets engrafted in the ACE occurs from the iris, which has a rich vessel-bed. When studying the dynamics of islet re-vascularization [6], at day 3 after transplantation, we observed that in the vicinity of sites where islets were attached to the iris, structural rearrangements of iris vessels took place. Seven days after transplantation, blood vessels continued to grow and by day 14 formed a micro-vascular network throughout the graft. From two to four weeks after transplantation, the vascular network became denser. By the end of four weeks, the network reached a plateau and was characterized by uniformly sized capillaries. The diameter of blood vessels started to decrease three days after transplantation and, by the end of four weeks, reached a diameter that was similar to that of the intra-islet vasculature in the pancreas. When transplanting islets freshly after isolation, these islets contain a substantial amount of intra-islet endothelial cells. These donor-islet endothelial cells contribute to the early events of the re-vascularization process by increasing the re-vascularization rate but do not increase the vascular density of the graft at four weeks after transplantation when compared to islets that were kept in culture and which had lost the majority of their endothelial cells [20]. Hence, both donor and host endothelial cells are capable of forming functional capillaries in the engrafted islets. As within the pancreas, islets engrafted in the ACE show a normal ultrastructure, i.e. endocrine cells and endothelial cells are separated by a single basement membrane and capillaries formed by thin endothelial cell bodies with fenestrations covered by a thin diaphragm [20]. The dynamics and the quality of the re-vascularization process are age-dependent [21]. Almaca et al. [21] demonstrated that islets of 18 months old mice showed the same blood-vessel density as islets from two months old mice; however, the aged islets had inflamed and fibrotic blood vessels. Moreover, the aged islets contained twice the number of macrophages, which were associated with blood vessels showing increased expression of intercellular adhesion molecule 1 (ICAM-1). In addition, these islets had higher expression levels of macrophage colony-stimulating factor receptor (CSFR1) and vascular cell adhesion molecule 1 (VCAM1). When transplanted into the ACE of two months old recipient mice, the re-vascularization of aged islets was delayed by one month when compared to young islets. Aged islets had lower vessel densities than younger islets in the first month after transplantation but showed noticeable re-vascularization within the following months. In addition to the delayed initial vascularization process, aged islets contained larger vessels that did not branch-out as much as vessels in young islet grafts. Between months three and seven after transplantation, aged islets showed regions of newly formed capillaries with smaller diameter, which allowed rejuvenation and functional recovery. It is noteworthy that transplantation of the

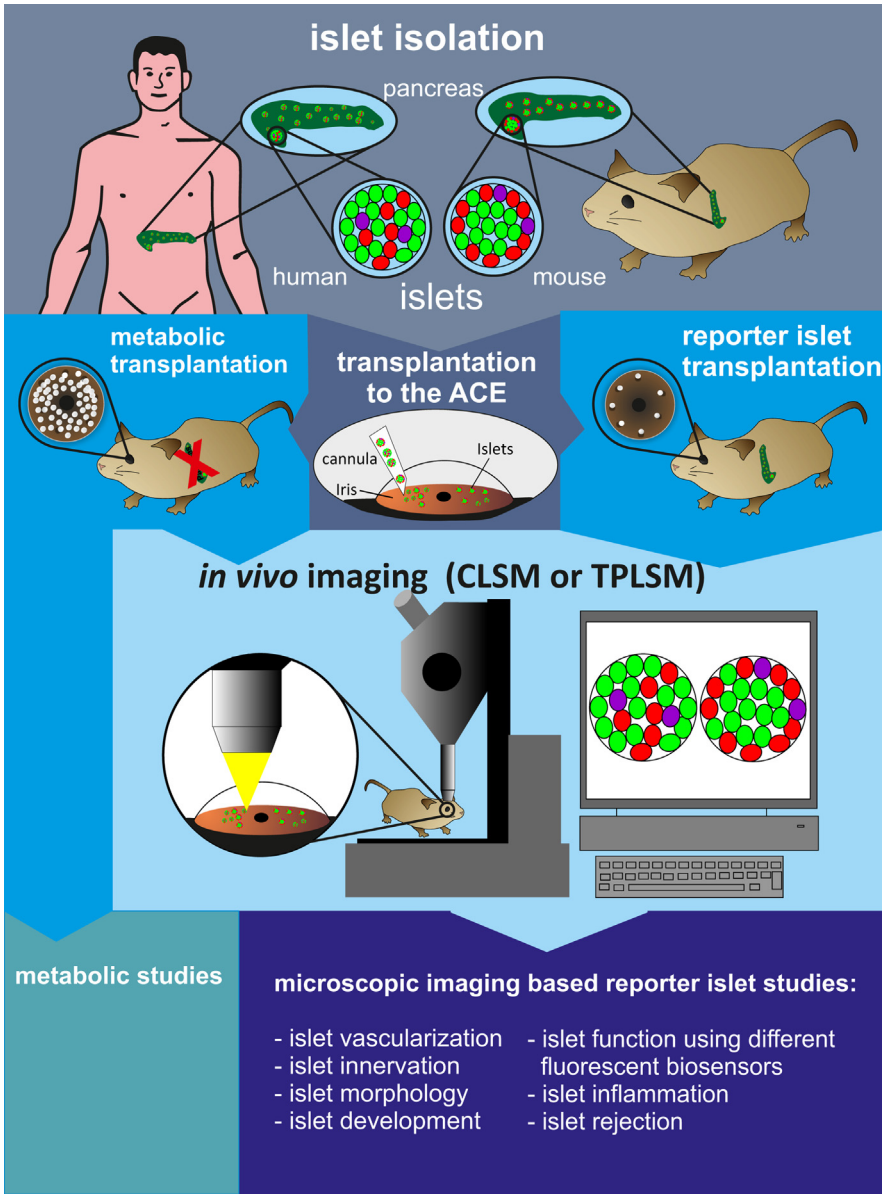

Figure 1: The anterior chamber of the eye (ACE) as a transplantation site for non-invasive and longitudinal monitoring of pancreatic islet/beta cell physiology and pathology. The endocrine pancreas consists of thousands to a million discrete micro-organs, i.e. islets of Langerhans, which are scattered throughout the pancreas. Each islet represents an entire micro-organ, containing the major hormone-producing cell types, i.e. insulin-producing beta cells (green), glucagon-producing alpha cells (red), and somatostatin-producing delta cells (purple). Noteworthy, human and rodent islets differ in their architecture, which has consequences for islet cell function. Following their isolation, pancreatic islets are transplanted into the ACE. In case of allo- or xeno-transplantation, immune-compromised animals serve as recipients. In the ACE, islets engraft on the iris, where they become vascularized and innervated. The cornea serves as a natural body-window, which allows non-invasive and longitudinal, microscopic, *in vivo* imaging at single-cell resolution by confocal laser-scanning microscopy (CLSM) or two-photon laser-scanning microscopy (TPLSM). Two general strategies for islet transplantation can be applied. The first is to use 'reporter islets' engrafted in the ACE for monitoring the status and function of the islets in the endogenous, *in situ* pancreas of the same animal. As a highly versatile research tool, this will allow for the identification of key-events in the development and progression of diabetes. Moreover, it will lay the foundation for a personalized medicine approach and will serve as a screening platform for new drugs and/or treatment protocols. In the 'metabolic transplantation,' the endogenous endocrine pancreas is replaced by a sufficient amount of islet mass transplanted to the ACE in combination with incapacitating the function of the endogenous islets. Here, engrafted islets in the eye control glucose homeostasis of the animal. This approach will allow establishment of islet-specific transgenic models, 'humanized' mouse models as well as provide the basis for a new clinical transplantation site for the cure of diabetes. Both strategies can be combined by using one eye of the animal for 'metabolic transplantation' while equipping the other eye with 'reporter islets' for microscopic functional analysis.

aged islets into the ACE of young recipient mice reversed the inflamed and fibrotic nature of the blood vessels. With regard to clinical islet transplantation therapy, this implies that pancreatic islets from elderly donors should rejuvenate in young recipients following engraftment. Moreover, the studies by Almaca and co-workers imply that functional properties of both human and mouse beta cells themselves do not change during aging and that the decreased function of aged islets is mainly caused by the local inflamed and fibrotic milieu [21]. This pinpoints the pancreatic islet vasculature as a potential target in the treatment of diabetes.

2.3. Islet innervation in the ACE

Pancreatic islets are innervated by the autonomous nervous system including sympathetic, parasympathetic and sensory fibers. While the overall parasympathetic input via acetylcholine leads to an increase in insulin release, the sum effect of the sympathetic input via epinephrine/adrenaline leads to a lowering of blood insulin concentration (reviewed in [22–24]). Similar to vascularization, islets transplanted into the ACE become innervated. The dynamics of islet re-innervation in the ACE have been demonstrated [16,25]. By performing immunohistochemical staining of islet grafts, Rodriguez-Diaz et al. [25] showed positive immunoreactivity for tyrosine hydroxylase (sympathetic) and for vesicular acetylcholine transporter (parasympathetic) as early as three days after transplantation. In fact, the time course of sympathetic and parasympathetic re-innervation followed that of re-vascularization. Two weeks after transplantation, axons could be seen inside islet grafts mostly associated with blood vessels. Between 15 and 30 days after transplantation, the number of axons along blood vessels increased as did their density, finally reaching a plateau 90 days post-transplantation. Sympathetic and parasympathetic densities in islet grafts in the ACE were similar to those observed in pancreatic islets. Like in the native pancreas, beta cells in the islet grafts in the ACE were innervated by the parasympathetic system. In the same study [25], we showed the feasibility to *in vivo* image islet innervation by transplanting islets into the ACE of mice expressing GFP in choline acetyltransferase-expressing neurons, i.e. ChAT-GFP mice [26]. The GFP-colored cholinergic neurons extended processes along the surface of the islet graft, a pattern that was similar to the *in situ* pancreas of these animals.

While it still has to be determined whether islets engrafted in the ACE are controlled by the same areas in the CNS as islets in the pancreas [27], this transplantation site offers the unique possibility to specifically and non-invasively modulate the input of the autonomous nervous system on islet function by either light illumination (alone or in combination with optogenetics) or by topical application of drugs (see section 2.4.2.).

2.4. 'Reporter islet' versus 'metabolic transplantation' concept

There are two general strategies for islet transplantation into the ACE. The first is to use islets engrafted in the ACE as reporters for the status and function of the islets in the endogenous, *in situ* pancreas of the same animal, i.e. the 'reporter islet' concept. The second approach is to replace the endogenous endocrine pancreas with a sufficient amount of islet mass transplanted to the ACE in combination with incapacitating the function of the endogenous islets. In the latter approach, the engrafted islets in the eye are responsible for controlling glucose homeostasis of the animal, hence the name 'metabolic transplantation'. Both strategies can be combined by using one eye of the animal for 'metabolic transplantation' while equipping the other eye with 'reporter islets' for microscopic functional analysis.

2.4.1. 'Reporter islet' concept

In the 'reporter islet' approach, a few islets transplanted into the ACE report on the functional status of the endogenous islets of the same animal in a longitudinal, non-invasive manner. This concept was introduced when analyzing the morphological changes of islets transplanted into the ACE of leptin-deficient ob/ob mice [28]. By comparing the morphology of islets transplanted into the ACE to that of endogenous islets of the same animal, our study showed that islets in the eye serve as optically accessible indicators of islet function in the pancreas. Islets engrafted in the ACE were monitored for several months in the living animal and parameters like islet volume, blood vessel size, and islet backscatter signal reported on the functional status of the islets. Moreover, longitudinal *in vivo* imaging of the 'reporter islets' in the eye revealed the reversed dysregulation of islets when these mice were treated with leptin. The latter observation indicates that the 'reporter islet' concept can be used to study the efficacy of specific treatment regimens on islet cell function and survival *in vivo*, non-invasively, and longitudinally.

Åvall et al. used the 'reporter islet' concept to verify that apolipoprotein CIII (apoCIII) locally produced in the islet, but not systemically produced by liver and intestine, affects islet function and survival [29]. We transplanted wild-type islets in one eye and islets from apoCIII-knockout mice in the other eye of the same animal and fed the mouse a high-fat-diet, exposing both types of islets to the same amount of high systemic apoCIII levels. Compared to islets from control mice, apoCIII-knockout islets (unable to produce apoCIII) were smaller in size and had lower levels of vessel density, macrophage infiltration, and NAD(P)H:FAD ratio, suggesting that the lack of intra-islet produced apoCIII prevents high-fat-diet-induced inflammatory reaction. This experimental set-up created a situation in which wild-type islets and genetically manipulated islets could be studied under the same experimental conditions.

These and other studies demonstrate the potential of islet intrinsic parameters for the study of islet/beta cell function and survival, namely islet volume, morphology of intra-islet vasculature including vessel diameter and blood vessel integrity (leakage), islet backscatter signal indicating islet integrity and beta cell insulin content, macrophage infiltration, collagen accumulation, cell death, and glucose metabolism indicated by NAD(P)H levels [6,7,29–33].

The high potential of imaging islets *in vivo* longitudinally with subcellular resolution was shown in studies that analyzed the dynamics of pancreatic beta cell insulin resistance [29,34]. Here 'reporter islets' were transduced with adenoviruses prior to their transplantation into the eye, encoding a genetically engineered fluorescent biosensor for insulin resistance. The biosensor was based on GFP-labeled FoxO1, a transcription factor that resides in the cytoplasm in insulin-sensitive cells but translocates into and accumulates in the nucleus of insulin-resistant cells. Employing the rat insulin-1 promoter in the expression construct restricted the expression of the biosensor to pancreatic beta cells. Beta cell insulin sensitivity was monitored in individual ob/ob mice from three months of age up to 10 months of age. *In vivo* imaging data revealed an increased nuclear localization of FoxO1-GFP at three months of age and a decrease of nuclear localization at 10 months of age, indicating beta cell resistance in the younger animals. The *in vivo* imaging data were supported by immunohistochemical analysis of endogenous FoxO1 localization in the *in situ* pancreata of these animals, thus validating the 'reporter islet' concept for this type of studies.

Chen et al. employed the 'reporter islet' concept to study beta cell compensation in mice during insulin resistance and pre-diabetes onset [35]. They performed longitudinal, *in vivo* imaging of reporter islets equipped with a Ca^{2+}-biosensor to report on beta cell mass and function during diet-induced progression towards pre-diabetes. In response to the dietary regimen, beta cells compensated whole body insulin resistance by increasing beta cell function and only to a lesser extent by increasing beta cell mass.

These studies exemplify the powerful potential of *in vivo* use of genetically engineered fluorescent biosensors for the study of islet/beta cell function and survival in health and disease. Ideally, such biosensors would cover all key-events in islet cell function. For pancreatic beta cells, such key-parameters would include glucose metabolism (ATP generation), plasma membrane depolarization,

intracellular Ca^{2+} handling (Ca^{2+} concentration, Ca^{2+} oscillations), insulin secretion, stimulus induced gene expression, beta cell insulin resistance, functional beta cell mass, beta cell identity, beta cell proliferation, and beta cell apoptosis among others. For *in vivo* monitoring, islets that were either transduced with a viral construct encoding the respective biosensor or islets that originate from a transgenic mouse expressing the biosensor in their beta cells can be used.

The large number of identified candidate genes combined with variations in environmental factors and life-style of each T2DM patient result in a heterogeneous and highly individual manifestation of the disease, requiring a personalized-medicine approach for diagnosis and treatment. Therefore, a better clinical understanding of the factors/processes involved in the regulation of islet cell/beta cell function and survival at the level of the individual diabetic patient is essential, especially in individual T2DM patients with very limited success of treatment there is a need for a non-invasive monitoring system that continuously reports on the status of the endocrine pancreas. In such a scenario, 'reporter islets' could consequently be of help to improve the treatment regimen.

2.4.2. Metabolic transplantation

In contrast to the 'reporter islet' concept, the metabolic transplantation protocol aims to replace the endogenous endocrine pancreas and to allow the islets engrafted in the eye to control systemic glucose homeostasis. This concept originated from the observation that 300 islets engrafted in the eye of mice that were rendered diabetic by streptozotocin treatment were sufficient to achieve normoglycemia in these animals [6]. In fact, when looking for the 'marginal islet mass' required to achieve normoglycemia, these studies showed that 150 islets are sufficient. This number of islets was used by Nyqvist et al. to evaluate the effect of freshly isolated versus cultured islets to revert diabetes following transplantation of islets into the ACE [20].

Performing metabolic transplantation with 200 islets, Almaca and co-workers showed that islets from 18 months old donors transplanted to two months old recipients were able to revert streptozotocin-induced diabetes; however, this took a month longer to achieve compared to islets from two months old donors [21]. Our data showed that this delay was not due to impaired beta cell function but caused by the fibrotic blood vessels and remodeled extracellular matrix of the aged donor islets. Interestingly, when engrafting aged islets in the two months old recipients, this was accompanied by a 'rejuvenation' of the islet blood vessel system which improved islet function and restored glycemia.

Rodriguez-Diaz et al. utilized metabolic transplantation to test the impact of autonomous innervation on islet function employing the pupillary reflex to light [25]. Here, we transplanted up to 300 islets either into the ACE or under the kidney capsule of mice rendered diabetic by streptozotocin. Stimulation with light constricts the pupil in response to increased cholinergic input while darkness leads to a dilation of the pupil in response to activated noradrenergic input. When exposing the mice with islet grafts to different light conditions, we found that fed mice with intra-ocular grafts that were changed from ambient light to darkness showed decreased insulin secretion and increased glycemia. Placing these mice back in ambient light lowered their blood glucose levels. In contrast, mice bearing islet grafts under the kidney capsule did not exhibit light-induced changes in glycemia. Similarly, the function of islets engrafted in the ACE was non-invasively manipulated by topical application of drugs affecting the autonomous nervous system, e.g. pilocarpine and atropine.

A special form of the metabolic transplantation concept is the generation of 'humanized' mouse models. Data by us and others have shown that human islets differ from rodent islets in the structure-function-relationship [6,25,36–38]. Therefore, it is of utmost importance to study human islet cell function and survival *in vivo*, under physiological conditions, during progression towards diabetes and finally in diabetes. Our own observations showed that the nature of the islet graft of the donor, e.g. characteristics of blood vessels and innervation pattern, is maintained after transplantation into the ACE. Since this also applies to human islets (unpublished data), this allows studying human islet biology/pathology *in vivo*. In this context, the ideal 'humanized' mouse is devoid of its own endocrine pancreas, which is replaced by a sufficient number of human islets to maintain normoglycemia in the recipient mice.

Abdulreda et al. employed a 'humanized' mouse model to study the effect of long-term, daily administration of recipient mice with liraglutide on human beta cell function [39]. In this study, we transplanted a marginal mass of human islets (500 islets) into the ACE of diabetic nude mice to accelerate human beta cell exhaustion and overall changes in glucose homeostasis. This set-up allowed studying the effect of long-term (250 days), continuous liraglutide treatment on the animal's metabolic state as well as the non-invasive, longitudinal monitoring of the human islets in the ACE. While in the initial phase treatment improved human islet function, prolonged treatment showed unexpected progressive deterioration in glycemic control associated with compromised insulin release and dysregulated glucose homeostasis, indicating the exhaustion of human islet cells.

To test the anterior chamber of the eye as a suitable transplantation site in non-human primates, Perez et al. performed a pilot study transplanting 20.000 islet equivalents (followed by a second transplantation of 18.000 islet equivalents 292 days post-transplantation) into the eye of a streptozotocin-diabetic baboon in combination with anti-CD154 monotherapy [40]. The ophthalmological examination showed no inflammation, no immune response, and no sympathetic ophthalmia. During the time of monitoring, i.e. 357 days, blood glucose homeostasis improved. Insulin C-peptide levels, evidence of insulin production, in the aqueous humor and in the circulation increased, and HbA1c values decreased, reflecting long-term glucose regulation. Intraocular islet grafts thus manifestly contributed to glucose homeostasis and improved glycemic control in this diabetic baboon.

3. THE ACE – A SITE TO STUDY IMMUNE REACTIONS IN REAL TIME *IN VIVO*

The exact etiology of both T1DM and T2DM are still not completely understood, but there is increasing evidence that islet inflammation is a common denominator in the development of both types of diabetes [41]. In T1DM, pancreatic beta cells are destroyed by an auto-immune-mediated process. Since one of the first observations using the ACE as a transplantation site was the 'immune-privileged' status of this site [8,42], this begged the fundamental question of whether the ACE is a suitable site to study immune reactions involved in diabetes. Therefore, it was of utmost importance to demonstrate that immune reactions can be studied in the ACE and that the involved mechanisms are the same as in other transplantation sites. Abdulreda and co-workers established an allo-transplantation model comparing the immune-rejection of allografts transplanted either into the ACE or under the kidney capsule [31]. This study demonstrated that immune responses against intraocular islet allografts were similar to those observed during immune-rejection of islets transplanted to the kidney. Transplantation of allografts into the ACE allowed for monitoring immune cell infiltration

and their interaction with target cells during progression of islet graft rejection. Noteworthy, this study demonstrated the possibility to track individual immune cells and quantify their kinetics and movement dynamics inside and outside the graft at different time points during progression of immune rejection.

Self-antigen-specific T-lymphocytes cause tissue damage in autoimmune diseases, such as T1D. Miska and co-workers established models of effective immune responses in the ACE versus native pancreas in terms of equivalent kinetics in tissue damage [43]. Employing the ACE and high-resolution imaging, we studied in real-time how self-antigen-specific T-cells interact with target cells *in vivo* and depicted the behavior of $CD4^+$ effector T-cells, $CD4^+$ regulatory T-cells, and $CD8^+$ effector cells. Besides demonstrating the powerful approach in obtaining data by non-invasive, longitudinal monitoring of immune reactions at the cellular level in the living animal, these studies demonstrated that immune privilege is broken when the grafts become vascularized. A detailed protocol for studying cytotoxic T-lymphocytes in the process of immune reaction in the ACE has been described [44].

To compare autoimmune destruction of allogeneic intraocular islet grafts versus endogenous islets, Mojibian and co-workers transplanted NOD-SCID islets into the ACE of NOD-SCID recipient mice. Following adoptive transfer of splenocytes from newly diagnosed NOD mice to the NOD-SCID recipient mice, Mojibian and colleagues monitored islet morphology and demonstrated that destruction of pancreatic islets in the eye mirrored that in the pancreas [45]. In a similar set-up, Schmidt-Christensen et al., following adoptive transfer, imaged the dynamics of $CD11c^+$ cells and $Foxp3^+$ cells in progressive autoimmune insulitis in islets engrafted in the ACE [46]. We demonstrated that, in spite of the initially immune privileged status of the eye, the ACE-transplanted islets developed infiltration and beta cell destruction, recapitulating the autoimmune insulitis of the pancreas. This was further exemplified by analyzing reporter cell populations expressing GFP under the Cd11c or Foxp3 promoters. We also provided evidence that differences in morphological appearance of subpopulations of infiltrating leucocytes can be correlated to their distinct dynamic behavior.

Employing a combination of elegant experimental set-ups, Chmelova and co-workers studied the potential of the remaining beta cell mass in response to immune intervention in the treatment of experimental T1DM [32]. By combining different protocols of adoptive transfer of immune cells with immune intervention, the authors studied morphological and functional changes of mouse and human islets during onset and remission of T1DM by non-invasive, longitudinal *in vivo* imaging of islets transplanted into the ACE of mice. Their data show significant loss of beta cell mass prior to onset of hyperglycemia, with beta cells being degranulated both by infiltrating immune cells and by increasing blood glucose levels. Immune intervention halted further destruction of beta cells but did not prevent their degranulation. Emerging hyperglycemia subsequent to immune intervention caused an increase in mouse beta cell mass by hypertrophy and proliferation, which stopped upon reaching normoglycemia. Human islets displayed a similar exhaustion and recovery during transient hyperglycemia. However, unlike mouse islets, human islets showed a reduced and transient increase in beta cell mass.

4. ANALYSIS OF ISLET/BETA CELL DEVELOPMENT

Two reports demonstrate the potential of the ACE to monitor early steps in pancreatic islet development. Although the ACE does not match the complex environment provided *in utero*, both reports show that pancreas development can be monitored longitudinally at single cell resolution. Ali and co-workers transplanted an E10.5 dorsal pancreatic bud from a MIP-GFP donor mouse into syngeneic recipients and monitored bud development over 21 days by microscopy [47]. GFP-positive cells from the MIP-GFP bud, i.e. mouse insulin-promoter-driven GFP, were observed four days after transplantation. At day seven after transplantation, these cells organized themselves into discrete cell clusters, which grew further during the full period of monitoring. Graft vascularization increased until day 10 after transplantation when it reached the levels of the native embryonic pancreas. Immunohistochemical analysis of the graft at 21 days after transplantation revealed the presence of exocrine cells, blood vessels, insulin-, glucagon- and somatostatin-producing endocrine cells, demonstrating that buds transplanted into the ACE recapitulate the development of the embryonic pancreas. Noteworthy, when removed at day 10 after transplantation from the ACE and stimulated by a forskolin-glucose cocktail, the ACE-transplanted buds responded with enhanced insulin secretion, which is in contrast to the *in vitro* cultured buds. These data suggest that at developmental stage E10.5, the intrinsic signals provided by the bud are sufficient to drive the differentiation of pancreas-specific cells, and this process might be facilitated by the rapid vascularization of the graft in the ACE.

Cras-Meneur and co-workers isolated mouse dorsal pancreatic buds at E13.0 and transplanted them into the ACE of CD1 albino recipient mice [48]. Using buds from mice that express YFP under control of the Pdx-promoter-driven Cre-recombinase allowed visualizing all cells derived from Pdx1 progenitors. Similar to the study by Ali et al., rudiments here also got vascularized, and the graft was easily imaged. However, the data in the study by Cras-Meneur and co-workers demonstrate a growth advantage of the mesenchyme leading to alterations in the differentiation pattern of the progenitors. A 90% mesenchyme depletion of the rudiments prior to transplantation into the ACE restored the differentiation process, which now mimicked that of normal pancreas development.

5. CONCLUSIONS – OUTLOOK

Data from different research groups applying the ACE model to address different tasks in experimental pancreatic islet research reveal the following important insights. Firstly, the nature of the islet graft of the donor is maintained in the ACE. This includes islet cell composition as well as patterns of vascularization and innervation and is true for rodent as well as human islets. Secondly, the milieu provided by the recipient affects the function of the engrafted islet and thus allows for studying the impact of a diabetic milieu, including immunological reactions and inflammatory processes, on rodent and human islets. Thirdly, in case of an autologous/syngeneic transplantation, the engrafted islets mirror the properties and function of *in situ* pancreatic islets and thus serve as 'reporter islets' successfully monitoring the efficacy of treatment protocols at the islet cell/beta cell level. Engrafted islets in the eye may also serve as an excellent model system to study diabetes complications. Finally, there are major differences in the structure/function relationship between rodent and human/non-human primate islets. Hence, to understand the complexity in human islet cell physiology and thereby identify novel druggable targets and drugs against these targets, studies must be performed with human islets *in vivo*.

The 'reporter islet' concept will allow the mapping of key-signaling events in the glucose-stimulus/insulin-secretion coupling during development and progression of T2DM in individual animals. This, combined with specifically designed intervention studies, will lay the

foundation for a personalized medicine approach in T2DM. In addition, 'reporter islets' will allow the development of screening platforms for new drugs and/or treatment protocols.

While the 'humanized' mouse will provide a convenient model for researchers in academia and industry to better understand human islet physiology and pathology in the living organism, this model will still have limitations due to the rodent physiology, e.g. species specifics in metabolism, provided by the host. Therefore, we have set-up the ACE imaging platform to study islet function in non-human primates. Following partial pancreatectomy, isolated islets were transplanted into the ACE of the same animal for functional analysis.

In 2011, we published a pilot study using a non-human primate to test the potential of the ACE as a transplantation site to cure T1DM [40]. The positive effects on glucose homeostasis in our rodent and monkey studies have raised the question of whether transplantation of islets to the ACE could ever be a clinical procedure for patients with T1DM. Thorough discussions with diabetologists, endocrinologists, and ophthalmologists regarding the anatomy of the human eye and our animal results showing no negative effects whatsoever on the eye have us to conclude that such studies will most likely be feasible, and we have indeed started to proceed in this direction. In this context, initial studies will be focused on safety involving patients who are legally blind from their diabetes and transplanted with a kidney, i.e. already on immunosuppressive drugs.

The pancreatic islet is a micro-organ consisting of several endocrine and non-endocrine cell types; the function of each cell type is affected by cell—cell interactions, autocrine, endocrine, and paracrine feedback loops as well as humoral and neural factors. By equipping individual cell types with fluorescent biosensors for functional read-out and combining this with novel modalities of cell-type-specific activation/inactivation by, for example, optogenetics, the ACE imaging approach will allow studying whole organ physiology/pathology in real time, longitudinally, at single cell resolution, and *in vivo*. This will allow for further understanding of the complexity of intra-islet communication. Moreover, combining this approach with selective genetic manipulation of tissues involved in the maintenance of glucose homeostasis will allow studying the crosstalk between islet cells and these tissues in the living organism in health and disease.

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CONFLICT OF INTEREST

P.-O.B. is cofounder of Biocrine AB, I.B.L. is consultant for Biocrine AB.

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