

Received Date : 26-Feb-2016

Revised Date : 17-Jun-2016

Accepted Date : 12-Jul-2016

Article type : Original Article

## **Key matrix proteins within the pancreatic islet basement membrane are differentially digested during human islet isolation**

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**Running title:** Human islet basement membrane digestion

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/ajt.13975

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# Accepted Article

## Abbreviations:

AK	Adenylate kinase
BM	Basement membrane
CIT	Cold ischaemia time
DBD	Donation after brain death
DCD	Donation after cardiac death
ECM	Extracellular matrix
HS	Heparan sulphate
HSPG	Heparan sulphate proteoglycan
IEQ	Islet equivalent
NP	Neutral Protease
ROI	Region of interest
r.t.	Room temperature
SBF-SEM	Serial block face-scanning electron microscopy
SI	Stimulation Index
TEM	Transmission electron microscopy

## Abstract

Clinical islet transplantation achieves insulin independence in selected patients, yet current methods for extracting islets from their surrounding pancreatic matrix are suboptimal. The islet basement membrane (BM) influences islet function and survival, and is a critical marker of islet integrity following rodent islet isolation. No studies have investigated the impact of islet isolation on BM integrity in human islets, which have a unique duplex structure. To address this, samples were taken from 27 clinical human islet isolations (donor age 41-59, BMI 26-38, CIT <10h). Collagen IV, pan-laminin, perlecan and laminin- $\alpha$ 5 in the islet BM were significantly digested by enzyme treatment. In isolated islets, laminin- $\alpha$ 5 (found in both layers of the duplex BM) and perlecan were lost entirely, with no restoration evident during culture. Collagen IV and pan-laminin were present in the disorganised BM of isolated islets, yet a significant reduction in pan-laminin was seen during the initial 24h culture period. Islet cytotoxicity increased during culture. Therefore, the human islet BM is substantially disrupted during the islet isolation procedure. Islet function and survival may be compromised as a consequence of an incomplete islet BM, which has implications for islet survival and transplanted graft longevity.

## Introduction

Clinical pancreatic islet transplantation has undergone huge advances over the past decade, with up to 85% of selected patients remaining insulin-independent for at least a year post-transplantation in leading centres worldwide and the majority of transplanted patients achieving resolution of life-threatening hypoglycaemic unawareness (1-5). Results are, however, significantly influenced by the number and quality of the human islets that are transplanted, with a minimum of 8000 Islet Equivalents (IEQ) per Kg required for consistent insulin-independence (6).

In addition, current methods for isolating human islets are suboptimal, with transplantable islet yields obtained only from a small proportion of pancreases donated into clinical programmes (7).

Optimising human islet isolation remains a priority to ensure consistent islet yields from the full range of donated pancreases (8). Indeed, the requirement to separate one intact organ (the islet) from within another organ (the pancreas), while maintaining structural integrity as well as viability, is a unique challenge.

To achieve optimal islet isolation requires delivery of collagenase blends to the pancreatic islet-exocrine interface, in order to digest the extracellular matrix (ECM) and release intact islets from the surrounding exocrine tissue. The development of such enzyme blends requires a detailed understanding of the molecular ultrastructure of the islet-exocrine interface, before and during pancreas digestion.

The islet-exocrine interface is comprised of an interstitial matrix and a basement membrane (BM). In addition to playing a structural role, there is increasing evidence to suggest that the islet BM influences  $\beta$ -cell insulin secretion and survival (9-11). BMs generally consist of a collagen IV network, which forms a scaffold into which the other BM components, including the laminins and heparan sulphate proteoglycan (HSPG) families integrate in a highly organised, supra-molecular architecture (12). The human islet BM contains numerous laminin isoforms (13), as well as perlecan, the predominant HSPG (14), which links laminins to collagen IV (15). Laminins and HSPGs are implicated in multiple signalling pathways, conferring downstream signals for optimal cell survival and function (11,12,16,17).

It is well established that cell-matrix detachment can lead to anoikis (18,19). During islet isolation, not only is the interstitial matrix destroyed, but the BM may also be partly digested. It is thought that the islet BM is a critical marker of islet integrity, with its disruption contributing to a reduction in islet number, viability, function and therefore, transplant success (11,20-22). Although loss of islet

BM during isolation and its subsequent remodelling following transplantation has been demonstrated in mouse islets (20), no studies have investigated this in human islets.

Importantly, the human islet BM is unique. It exists as a duplex structure, consisting of a vascular BM associated with the islet capillary, in addition to an endocrine BM integral to the islet itself (13). The composition of each BM layer differs, but laminin- $\alpha$ 5 is present in both layers (13) and can thereby be used as a marker for the integrity of the duplex BM. Mouse islets have only a single BM. This fact, along with substantial differences between the mouse and human in both islet structure and islet isolation procedures, suggests that a study investigating islet BM digestion in human islets is long overdue.

Using current isolation methods, higher islet yields often result from donor pancreases that are “sub-optimal” with regards islet function and longevity, i.e. pancreases from older donors with high BMIs (23,24). It is hypothesised this is due to the “looser” structure of the pancreatic ECM of these donors, compared with their younger, leaner counterparts. However, the function of islets isolated from younger donors is significantly better (24-26), yet it remains difficult to consistently isolate transplantable yields from these donors. Knowledge of the composition and structure of the islet-exocrine interface (which is the “substrate” for digestion), even in “standard” (older, higher BMI) donors, is relatively incomplete, despite this donor group comprising around 55% of pancreases accepted for islet isolation in our centre (27). Characterisation of the human islet BM will help optimise the efficiency of the digestion phase of human islet isolation, which will benefit islet yield, function and survival, thereby maximising isolation success from the full range of donor pancreases.

The aim of this study was to characterise the digestion of key human islet BM components in pancreases from donors that fulfil the criteria for clinical transplantation (marginal donors were excluded). To do this we studied BM composition of (i) islets *in situ* in the human pancreas, (ii) islets immediately following isolation and (iii) cultured islets prior to transplantation.

## **Materials and Methods**

### **Islet isolation and sampling technique**

With appropriate consent and ethical approval, human pancreases (n=27) were procured from multi-organ donors (Table 1). Islets were isolated using conventional methods comprising intraductal enzyme infusion using Collagenase NB1 (2101-2965 PZU) and Neutral Protease NB (NP; 50 DMCU; Serva, Heidelberg, Germany), followed by semi-automated digestion and continuous density gradient purification (28,29). 0.5cm<sup>3</sup> tissue samples were taken from the pancreas body and snap frozen in liquid nitrogen. Islet samples were fixed in 4% paraformaldehyde (Sigma, Poole, UK) or placed into hypotonic buffer with 1% protease inhibitor cocktail (constituents of Nuclear Extraction Kit, Actif Motif, Carlsbad, CA, USA), immediately following purification ("pre-cultured" sample), and after 24h, 48h and 72h of culture at 37°C in CMRL-1066 medium (PAA Laboratories, Pasching, Austria) supplemented with 2% Human Serum Albumin (HSA; BPL, Elstree, UK), 10000U/ml Pen-Strep and 2mmol L-Glutamine (both Lonza, Basel, Switzerland). Although 27 islet preparations were used in the study, samples were not collected from each stage in all preparations.

### **Islet viability**

Viability was measured post-isolation, and at 24h, 48h and 72h culture for a subset of islet preparations, by incubating a small sample of islets for 3 minutes with fluorescein diacetate (0.1mg/ml; Sigma) which stains live cells green and ethidium bromide (0.1mg/ml; Sigma), a red dye which can permeate only cells with damaged membranes. The percentage of dead cells per islet was approximated by visual enumeration using a fluorescent microscope.

### **Islet cytotoxicity**

Cytotoxicity was assessed in cultured islets. Supernatant samples were collected pre-culture and at 24h, 48h and 72h of culture and stored at -25°C. The release of adenylate kinase (AK) from damaged

cells was measured using a ToxiLight bioassay kit (Lonza), according to the manufacturer's instructions. Results are expressed as percentage of control (pre-cultured samples).

### **Islet function**

*In vitro* islet function was assessed in triplicate during static incubation (30). 10-12 hand-picked and size-matched islets per tube were preincubated in Krebs-Ringer bicarbonate buffer (KRBB) supplemented with 2mg/ml BSA (Sigma) and 1mmol/L glucose (Sigma) for 1h at 37°C, followed by 1h incubation in KRBB with 20mmol/L glucose at 37°C. Insulin secretion was determined by radioimmunoassay (Millipore, Livingstone, UK). Stimulation index was calculated by dividing insulin release at 20mmol/L glucose by insulin release at 1mmol/L glucose.

### **Enzymatic treatment and immunolabelling of tissue sections**

Pancreatic tissue sections (10µm) were prepared using a Cryostat (Leica, Milton Keynes, UK), collected onto Superfrost glass slides (Thermo-Fisher Scientific, Loughborough, UK) and stored at -25°C. Sections were incubated for 2 or 5 minutes at 37°C with clinically comparable concentrations of Collagenase (6.56 PZU/ml) and NP (0.14 DMCU/ml) or with HBSS (control), then treated with cold running water to stop enzymatic activity. Sections were fixed with 2.5% paraformaldehyde, blocked with 5% goat serum (Vector Laboratories, Peterborough, UK) for 1h, then incubated with primary antibodies for 1h at room temperature (r.t.): polyclonal rabbit anti-pan-laminin, polyclonal rabbit anti-collagen IV, monoclonal mouse anti-perlecan and monoclonal mouse anti-laminin-α5 (all Abcam, Cambridge, UK) along with polyclonal guinea pig anti-insulin (DAKO, Ely, UK). Sections were then treated with secondary antibodies: AlexaFluor488-conjugated goat anti-rabbit (Thermo-Fisher Scientific), CFL488-conjugated goat anti-mouse (Santa Cruz Biotechnology, Dallas, TX, USA) and texas red-conjugated goat anti-guinea pig (Vector Laboratories) for 0.5h at r.t., then mounted using Vectashield HardSet mounting media (Vector Laboratories).

## **Quantitative analysis of islet BM components during digestion**

A minimum of 6 islets per donor per condition were imaged using an Axioskop 40 light microscope (Carl Zeiss, Jena, Germany). 8-bit black and white images were taken using Axiovision software. A semi-automated, quantitative measurement of the area and fluorescent intensity of each islet was carried out using an in-house macro in ImageJ. Islet area was exclusively selected as the region of interest (ROI) using the insulin staining signal. Peri- and intra-islet protein area and fluorescent signal above the defined threshold were measured within the ROI. Protein area was normalised against islet area, then multiplied by the mean signal intensity, with the treatment data further normalised against the controls.

## **Islet immunolabelling**

Whole islets were immunofluorescently labelled to determine BM protein localisation. Following 3 washes in PBS containing 0.05% Tween (Sigma), islets were blocked with 5% goat serum and permeabilised with 0.1% Triton (Sigma) for 0.5h at r.t. Islets were incubated with primary antibodies as above for 2h, then secondary antibodies as above for 1h. Islets were viewed and images captured using a Zeiss LSM510 confocal laser scanning microscope. The quantity of BM protein was estimated by visual enumeration using a numerical scoring system, from 0 (no protein present) to 3. An average of 8 islets were scored per sample.

## **Protein extraction and quantification**

Protein was extracted from islets by homogenisation in ice-cold radioimmunoprecipitation assay (RIPA) buffer (Sigma). A BCA assay was used to quantify total protein, according to the manufacturer's instructions (Thermo-Fisher Scientific).

## **ELISAs**

To quantify the amount of BM protein at each time point, islet lysates were analysed using ELISA kits specific for pan-laminin (Abcam), collagen IV, laminin- $\alpha$ 5 and perlecan (all USCN Life Science,



Wuhan, China) according to the manufacturer's instructions. 10µg protein was loaded per well.

Results are expressed as ng/ml.

### **Transmission Electron Microscopy (TEM) and Serial Block Face-Scanning Electron Microscopy (SBF-SEM)**

Isolated islets (n=3) and intact pancreatic tissue samples (n=3) were fixed and processed using a high density staining method suitable for block face imaging as previously described (31,32). For further details see Supplemental Materials and Methods.

### **Statistical analysis**

Data are expressed as mean±SEM. Multiple experimental conditions were compared using linear mixed modelling (for slide-based digestion experiments) and one-way ANOVA followed by post-hoc Tukey's or Kruskal-Wallis tests, with post-hoc Dunn's test for multiple comparisons (for islet viability, cytotoxicity, immunolabelling and ELISAs). Correlation analysis assessed associations between islet BM loss and donor and islet isolation parameters, with Pearson or Spearman correlation coefficients used as appropriate.  $p<0.05$  was considered significant. The software packages Prism-Graphpad and SPSS Statistics were used.

## **Results**

Islet isolations were performed on 27 pancreases from "standard" donors (median age=52, range 41-59; median BMI=30, range 26-38) accepted for clinical islet transplantation. Yield was 209,000±18,000 IEQ, purity was 61±3% and viability was 77±2% (Table 1).

### **Composition of the human islet BM**

Collagen IV, pan-laminin, perlecan and the laminin-α5 isoform were found to be major components of the BM of islets *in situ* within the human pancreas (Figure 1a). Enzyme treatment of pancreas

sections resulted in significant digestion of collagen IV and pan-laminin in the islet BM (Figure 1a,b;  $p<0.05$ ). A  $35\pm 8\%$  reduction in the quantity of laminin- $\alpha 5$  in the islet BM was seen after 2 minutes of enzyme treatment, with a  $45\pm 6\%$  reduction observed following 5 minutes treatment (Figure 1a,b;  $p<0.05$ ). Perlecan was most susceptible to enzyme treatment, with significant digestion observed following both 2 ( $53\pm 7\%$  reduction) and 5 minutes ( $67\pm 6\%$  reduction) treatment (Figure 1a,b;  $p<0.05$ ). Immunolabelling control experiments showed no background signal or cross-reactivity between antibodies (Figure S1).

### **Localization of BM proteins in isolated islets**

Immunostaining of pre-cultured islets revealed a total loss of perlecan and laminin- $\alpha 5$ , indicative of substantial BM damage during islet isolation (Figure 2c,d). Collagen IV and pan-laminin labelling was, however, present in pre-cultured islets and appeared to follow the intra-islet capillary tracts (Figure 2a,b). A  $46\pm 14\%$  reduction in pan-laminin labelling over the first 24h of culture was seen ( $p<0.01$ ), with expression then remaining stable for the final 48h culture period (Figure 2b,f,h,j,k). A trend towards a reduction in collagen IV over time was found ( $36\pm 16\%$  reduction 0-48h culture,  $p=ns$ ; Figure 2a,e,g,i,k), with labelling for both perlecan and laminin- $\alpha 5$  remaining absent throughout the 72h culture period (Figure 2k, images not shown).

### **Quantification of BM proteins**

The results generated by ELISA represent the total amount of protein present in the islet preparation, regardless of whether the protein is present in islets themselves or in acinar tissue, and is influenced by the purity of the sample. Therefore, all ELISA results have been corrected for the purity of each respective islet preparation. Despite this, it is important to consider the results of immunostaining and ELISAs in parallel.

A  $62\pm 10\%$  reduction in pan-laminin during the first 24h of culture was found ( $8.6\pm 1.8$  vs  $3.3\pm 0.9$ ng/ml, pre-cultured vs 24h culture,  $p<0.05$ , Figure 3). The quantity of pan-laminin present at 48h and 72h was also significantly reduced compared to pre-cultured islets ( $p<0.01$ ). Collagen IV was

unchanged over the 72h culture period (Figure 3). Quantities of laminin- $\alpha$ 5 and perlecan were around the lower detection limits of the respective ELISAs, correlating with the lack of positive labelling seen using islet immunostaining.

### **Ultrastructural assessment of the islet BM**

In the intact pancreas, islets are enclosed in an encapsulating BM structure (Figure 4a,i). TEM reveals the double leaflet organization of the BM and close association of the islet with extra-islet capillaries (Figure 4a). Collagen fibres appear uniform in their organization without crosslinking. By contrast, no BM structure is visible in isolated islets (Figure 4a,ii). In order to further define the loss of islet BM, SBF-SEM was used to serially section isolated islets (Figure 4b). Through 100nm serial sections, no organization to the islet BM was identified (Figure 4b,ii).

### **Effect of islet BM disruption on islet survival**

During culture, no change in islet viability was observed (Figure 5a), yet assessment of islet cell cytotoxicity showed an increase in the release of AK (Figure 5b). This increase in cytotoxicity mirrors the loss of pan-laminin from islets over time in culture (shown in Figures 2,3), and may result from the extensive loss of perlecan and laminin- $\alpha$ 5 seen during the digestion process (shown in Figure 2c,d).

### **Correlation of islet BM loss with donor and islet isolation parameters**

Correlations were performed to assess associations between donor and islet isolation parameters and loss of pan-laminin in the islet BM. As perlecan and laminin- $\alpha$ 5 were lost entirely in all islet preparations studied and collagen IV was present post-isolation, remaining stable during culture, correlation analysis was not possible for these datasets. Pan-laminin content of pre-cultured islets showed a negative correlation with pancreas cold ischaemia time (CIT;  $r=-0.75$ ,  $p<0.01$ ; Figure 6a). The pan-laminin content of 24h cultured islets was negatively correlated with pancreas CIT ( $r=-0.75$ ,  $p<0.01$ ; Figure 6b) and positively correlated with islet yield (islet number;  $r=0.72$ ,  $p<0.05$ ; Figure 6c).

## Discussion

The fate of human islet BM components following islet isolation has not been reported, despite previous studies demonstrating BM loss in rodent islets (20). This study, using *in vitro* digestion experiments combined with analysis of intact pancreatic tissue, isolated and cultured islets, has determined that key components of the human islet BM are differentially digested during islet isolation, and that the BM fails to recover during islet culture prior to transplantation.

Our results confirm and extend those of Virtanen *et al.* (13), demonstrating the presence of collagen IV and laminin- $\alpha$ 5 in both layers of the islet double BM. Importantly, our study found for the first time that both laminin- $\alpha$ 5 and perlecan are lost entirely in isolated islets, indicating that extensive BM disruption occurs during islet isolation, which has potentially major translational significance.

Laminin- $\alpha$ 5 and perlecan appear particularly sensitive to digestive enzyme treatment and this apparent breach of the endocrine BM layer may severely compromise islet cell function and survival. The disorganised islet BM that remains following islet isolation contains collagen IV and pan-laminin. Substantial remodelling or restructuring did not occur during culture, with a decrease in pan-laminin observed during the initial 24h of culture and collagen IV remaining stable. No recovery of laminin- $\alpha$ 5 or perlecan was evident during culture. Cytotoxicity increased during culture, echoing the loss of laminin from cultured islets, thereby suggesting that BM disruption and loss of laminins and perlecan may have a deleterious impact on islet survival.

Loss of laminin- $\alpha$ 5 during culture has been reported in isolated canine islets (33). However, perlecan was found to be absent in isolated mouse islets (20), which is in agreement with our results in human islets. Mouse islets lose collagen IV during isolation, with no recovery during culture (20), yet our results show the presence of collagen IV in isolated human islets. This inconsistency between

mouse and human islets is likely due to considerable differences in BM structure between species (13) and in islet isolation procedures.

Our results indicate that the BM is not lost as a whole at the same rate, but instead that certain components are more vulnerable to digestion. This could be due to differing effects of each component of the collagenase enzyme blend or that the loss of surrounding tissue affects the integrity of specific proteins more than others. In the BM, members of the laminin family bind to the cell surface through interactions with their cellular receptors. Laminins are linked to the collagen IV network via collateral interactions with perlecan and nidogen, forming a highly structured BM (15). Disruption of any component will compromise BM integrity. Furthermore, an incomplete BM will potentially expose proteins originally hidden within the specialised architecture, allowing access of digestion enzymes and further BM breakdown. The two layers of the human islet BM have distinctly different organisation (13) and so would be expected to differ in their susceptibility to digestion.

Therefore, it will be important to investigate the loss of additional BM components, such as nidogen (20) and other laminin isoforms (13). Analysis of the islet BM during and post-islet isolation will enable the effective evaluation of digestion enzymes, with studies underway in our laboratory to determine the exact substrates of each component of the clinically-used enzyme blends.

Identification of matrix proteins unique to the peri- and intra-islet BM will help the development of novel, targeted enzyme blends. Laminin- $\beta$ 2 is prominent only in the intra-islet vascular BM, with laminin- $\alpha$ 2 comprising the BM of exocrine cells only (13). Laminin- $\alpha$ 2 could therefore represent a target for tailored enzymes, which would cleave only the specific matrix proteins surrounding the islet, thereby freeing islets from their supporting exocrine tissue, whilst leaving important intra-islet BMs intact.

Many clinical islet isolation centres, including our own, have adopted a short period of islet culture (<48h) prior to transplantation (34). This provides an opportunity for islet quality assessment, recipient matching, transport of islets and recipients to the transplant centre, and initiation of

immunosuppressive protocols. However, integral to this culture period is the risk of islet loss. It was therefore important to study the effect of culture on the islet BM. Isolated islets retained collagen IV, but pan-laminin was lost during culture. This contrasts with the extensive digestion of laminin- $\alpha$ 5 and perlecan during islet isolation, with no evidence of restoration during culture. It is important to note that, in our centre, islet culture conditions prior to transplantation are minimal in composition (CMRL media supplemented with 2% HSA and 2mmol L-Glutamine). Studies are underway in our laboratory to investigate whether modifications to culture conditions, including supplementation with human AB Serum and combinations of matrix proteins, will enable the preservation and recovery of islet BM and thus improve islet function and survival. It will also be necessary to investigate recovery and remodelling of the human islet BM post-transplantation and its impact on islet engraftment using an *in vivo* model.

BMs provide a barrier function, structural support and vital signalling for adjacent cells. The majority of  $\beta$ -cells in native islets are in contact with BM, therefore its preservation is crucial for maintaining optimal islet function (22). Laminins interact with islet cells via receptors including integrins (13,35) and Lutheran glycoproteins (13), conferring downstream biochemical pathways (12,16,36). Integrins  $\alpha$ v $\beta$ 3 and  $\alpha$ v $\beta$ 5 regulate adhesion and differentiation, with  $\alpha$ 3 $\beta$ 1 and  $\alpha$ 6 $\beta$ 1 involved in insulin secretion (37-39). Notably, our study found a positive correlation between islet yield and laminin content following 24h culture, suggesting that substantial loss of laminin and other components of the BM may impact islet survival pre-transplantation. Heparan sulphate (HS) has recently been shown to play a role in  $\beta$ -cell survival and differentiation (40,41) and in protection against reactive oxygen species-induced damage (17,40). HSPGs, including perlecan, are composed of HS chains attached to individual core proteins (42-46). During mouse islet isolation HS is lost and fails to recover during culture (17), yet importantly, antioxidant treatment preserved HS during islet isolation, resulting in a significant improvement in islet survival (17). This suggests that loss of perlecan during human islet isolation, as demonstrated in our study, may substantially impact islet viability and function, indeed, the extensive loss of perlecan was evident in all islet preparations

studied, with the mean viability being  $78 \pm 2\%$ . Therefore, the use of antioxidant treatment for preservation of HS during human islet isolation warrants further investigation.

Pancreas shortage is a primary factor preventing islet transplantation from being made available to more patients. It is therefore increasingly common for pancreases from marginal donors to be offered for islet transplantation, yet isolation results from these pancreases are frequently suboptimal. This study could be extended to include marginal donors, for example pancreases from young donors and donation after cardiac death (DCD) donors. This knowledge will help optimise and tailor the islet isolation process for the full range of donors, thereby increasing the available pancreas donor pool. Caution is however needed, as our results suggest that pancreas CIT influences the susceptibility of pan-laminin to digestion during islet isolation, with islets isolated from pancreases with shorter CITs retaining a greater quantity of pan-laminin. Islets from higher yielding isolations appear to retain greater quantities of pan-laminin following 24h culture, suggesting that loss of laminin during islet isolation and culture could be contributing to poorer isolation outcomes with reduced islet survival.

The current focus on developing islet scaffolds, both pre- and post-implantation (47-55), means a detailed understanding of the normal molecular structure of the pancreas is important. One aim of such scaffolds is to support islet survival by providing essential cell-to-matrix interactions, which may be lost during islet isolation. By characterising the impact of islet isolation on the human islet BM, this study has contributed significant data that will aid in the development of islet scaffolds.

The loss of BM may negatively impact islet survival and be a contributory factor to the need for excessively large numbers of islets that are required for islet transplantation to be successful. This study has characterised the human islet BM and its disruption during islet isolation, in pancreases from the “standard” donor, typically accepted for islet isolation. We aim to extend our investigations to study the islet BM in the full range of donor pancreases. This detailed characterisation will have

huge translational potential, for optimising digestion enzyme blends and isolation techniques, as well as for the development and bioengineering of scaffolds and encapsulation technologies.

## **Acknowledgments**

This study was supported in part by the Diabetes Research and Wellness Foundation (DRWF) and the National Institute for Health Research (NIHR) Oxford Biomedical Research Centre Programme.

This work was carried out in the Nuffield Department of Surgical Sciences and the Oxford Centre for Diabetes, Endocrinology and Metabolism, both at the University of Oxford. The authors would like to thank the members of the Oxford Human Islet Isolation Team for their excellent work in the isolation facility, Ms Emma Pope for technical assistance and Alex Mironov and Toby Starborg from the Electron Microscopy Core Facility (Faculty of Life Sciences, University of Manchester) for their support.

SEC designed the project. SEC, RHV, AJW, AJM, AAA, BH, JDJ, EM, PAB, RDR and MJD performed the experiments, collected and analysed the data. PR, KEK and PRVJ provided experimental facilities and equipment. SEC, MJD and PRVJ prepared the manuscript. MJD, PRVJ and SJH contributed to the design and overall direction of the project.

## **Disclaimer**

This research was supported by the NIHR Oxford Biomedical Research Centre Programme. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.



## Disclosure

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

## Figure legends

**Figure 1:** Differential digestion of islet BM components following *in vitro* slide-based digestion analysis.

a. Following treatment of pancreas sections with a clinically used collagenase and NP blend for 2 or 5 minutes, digestion of the key BM proteins collagen IV, pan-laminin, laminin- $\alpha$ 5 and perlecan (*green*) is seen around and within the islet (*red*, insulin labelling of  $\beta$ -cells). Scale bars = 100 $\mu$ m.

b. Quantitative analysis of digestion. The intensity of labelling for each specific protein following 2 and 5 minutes of enzyme treatment was normalised to respective controls. All four BM components were significantly digested following 2 and 5 minutes of digestion enzyme treatment (\*  $p < 0.05$  compared to control). Data represent the mean  $\pm$  SEM from  $n = 9$ -16 islet preparations. BM, basement membrane; NP, neutral protease.

**Figure 2:** Islet BM is lost during the islet isolation process.

a-j. Representative images of whole isolated islets immunofluorescently labelled for collagen IV (a,e,g,i), pan-laminin (b,f,h,j), laminin- $\alpha$ 5 (c) or perlecan (d)(all *green*) and insulin (*red*), over a 72h period of culture. Scale bars = 100 $\mu$ m.

k. The amount of BM protein present at each time point was scored visually. Significant differences in pan-laminin content were seen between pre-cultured islets and cultured islets (\*\*  $p < 0.01$  vs pre-cultured islets). Although there was a trend towards a reduction in collagen IV over time, this did not

reach significance. Data represent each individual islet isolation, along with mean $\pm$ SEM from n=8-17 islet preparations. BM, basement membrane.

**Figure 3:** Loss of BM components during islet culture, quantified by ELISA.

Graph shows the amount of collagen IV and pan-laminin present in isolated islet preparations over 72h in culture, as measured using ELISA. The level of collagen IV remained stable over the entire culture period. A significant reduction in pan-laminin expression compared to pre-cultured islets was seen at each timepoint (\*  $p<0.05$ , \*\*  $p<0.01$ , \*\*\*  $p<0.001$  vs pre-cultured islets). A trend towards the further loss of pan-laminin after the initial 24h culture period was seen, but did not reach significance. Data are mean $\pm$ SEM, corrected for purity, from n=10-15 islet preparations. BM, basement membrane.

**Figure 4:** TEM and SBF-SEM analysis of the human islet BM pre and post islet isolation.

Panel A (i) illustrates a typical TEM image of the edge of an islet in the intact pancreas. Islet cells are labelled and the boundary with the exocrine pancreas is clearly demarcated with a collagen-rich capsule composed of uniform fibres. Data are representative images of islets from n=3 pancreases.

Panel A (ii) illustrates a typical isolated islet boundary, which is lacking a BM. The image shows a  $\beta$ -cell, clearly identifiable through the dense core secretory granules. Data are representative images of preparations of isolated islets obtained from n=3 donors. Scale bar = 1 $\mu$ m.

B. Isolated islet ultrastructural images were generated using SBF-SEM. Panel B (i) is a single section of the tissue stack. Panel B (ii) is a digital reconstruction of the image stack which comprised a total of 293 serial sections of tissue in 100nm thicknesses. A section through the serial stack at serial section 160 in the Z-plane is also shown. Note in all images that there is no BM structure associated with the isolated islets. Images are representative of islets obtained from n=3 donors. Scale bar = 10 $\mu$ m. BM, basement membrane.

**Figure 5:** Islet viability and cytotoxicity during culture.

- a. No change in islet viability compared to pre-cultured islets was seen up to 72h of culture ( $p=ns$ ).
- b. Islet cell cytotoxicity increased over time in culture. A significant increase in cytotoxicity compared to pre-cultured islets was found at 72h culture (\*\*  $p<0.01$ ) and between 24h and 72h cultured islets (\*  $p<0.05$ ). Data are mean $\pm$ SEM from  $n=9$  islet preparations.

**Figure 6:** Correlations of islet pan-laminin content with donor and islet isolation parameters.

- a. Pan-laminin content of pre-cultured islets vs CIT,  $r = -0.75$ ,  $p<0.01$ . b. Pan-laminin content of 24h cultured islets vs CIT,  $r = -0.75$ ,  $p<0.01$ . c. Pan-laminin content of 24h cultured islets vs islet number,  $r = 0.72$ ,  $p<0.05$ . Pan-laminin content of pre-cultured and 24h cultured islets was measured by ELISA.  $n=9-14$  islet preparations.

## Supporting information

Additional Supporting Information may be found in the online version of this article.

### Supplemental Materials and Methods

**Figure S1:** Immunofluorescence labelling controls.

Pancreas sections were stained with the following combinations of antibodies. BM protein labelling (*green*), insulin labelling (*red*). (A) Single stain for BM protein only: i. Rabbit anti-collagen IV IgG + goat anti-rabbit Alexa488, ii. Rabbit anti-pan laminin IgG + goat anti-rabbit Alexa488, iii. Mouse anti-laminin- $\alpha 5$  IgG1 + goat anti-mouse CFL488, iv. Mouse anti-perlecan IgG1 + goat anti-mouse CFL488. (B) Single stain for insulin: Guinea pig anti-insulin IgG + goat anti-guinea pig Texas Red. (C) Primary antibodies towards BM proteins with goat anti-guinea pig Texas Red secondary antibody: i. Collagen IV, ii. Pan-laminin, iii. Laminin- $\alpha 5$ , iv. Perlecan. (D) Secondary antibodies alone: i. Goat anti-guinea

pig Texas Red + goat anti-rabbit Alexa488, ii. Goat anti-guinea pig Texas Red + goat anti-mouse CFL488.

(E) Primary antibodies alone: i. Collagen IV + Insulin, ii. Pan-laminin + Insulin, iii. Laminin- $\alpha$ 5 + Insulin, iv. Perlecan + Insulin. f. Insulin primary antibody with anti-rabbit or anti-mouse secondary antibodies: i. Insulin + goat anti-rabbit Alexa488, ii. Insulin + goat anti-mouse CFL488. (G) Insulin primary antibody with anti-guinea pig and anti-rabbit or anti-mouse secondaries: i. Insulin + goat anti-guinea pig Texas Red + goat anti-rabbit Alexa488, ii. Insulin + goat anti-guinea pig Texas Red + goat anti-mouse CFL488. (H) Normal rabbit IgG or normal mouse IgG1 with anti-guinea pig and anti-rabbit or anti-mouse secondaries: i. Normal rabbit IgG + goat anti-guinea pig Texas Red + goat anti-rabbit Alexa488, ii. Normal mouse IgG1 + goat anti-guinea pig Texas Red + goat anti-mouse CFL488.

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## Tables

Table 1. Donor and islet isolation characteristics

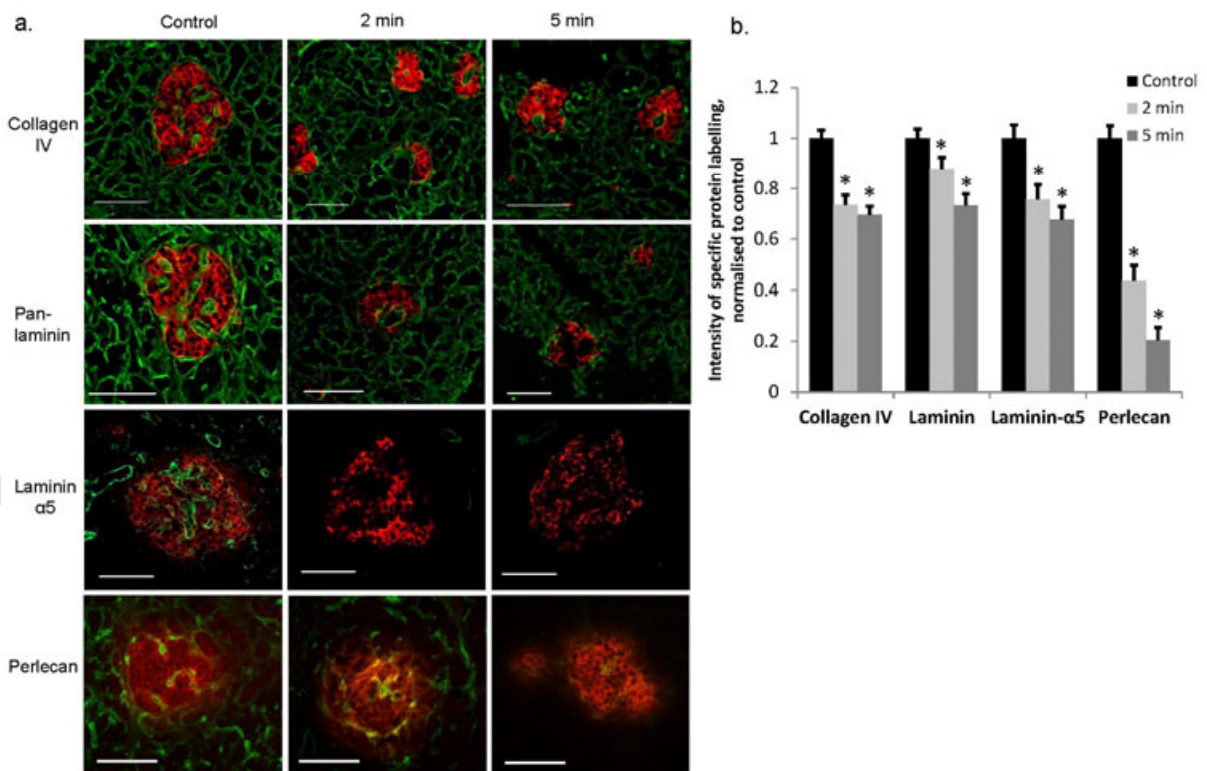
Age (y)	Sex	BMI	CIT (h)	Pancreas weight (g)	Donor type <sup>a</sup>	Yield (islet number x10 <sup>3</sup> )	Yield (IEQ x10 <sup>3</sup> )	Purity (%)	Viability (%)	Function (SI)
53	F	31	5	82	1	130	278	90	85	-
59	F	29	7.5	90	1	85	170	60	75	-
44	M	30	6.5	73	1	505	167	50	85	-
57	F	29	8	75	1	80	140	70	-	1.5
51	F	32	7.5	110	1	50	200	40	-	-
54	F	28	7	87	1	190	265	60	90	8
50	M	31	5	125	1	140	410	50	85	-
58	M	29	5	95	1	33	134	70	-	-
51	M	30	6	126	2	95	250	60	85	-
42	F	29	6	72	1	133	330	50	70	-
51	F	33	7	113	1	54	235	50	-	-
54	M	38	9.5	103	1	65	147	40	-	3.2
44	F	29	4	87	2	33	160	50	-	2.6
45	F	36	6	70	1	35	97	50	78	-
53	M	38	4	76	1	85	180	50	74	-
52	F	34	8	80	1	69	149	50	62	-
53	F	30	5	101	1	72	166	70	76	2.6
55	M	27	6	80	1	52	84	75	80	-
56	F	30	5.5	70	1	177	280	75	71	3
41	F	35	6.5	92	1	101	239	60	75	10
52	M	31	6	116	1	275	300	60	75	2



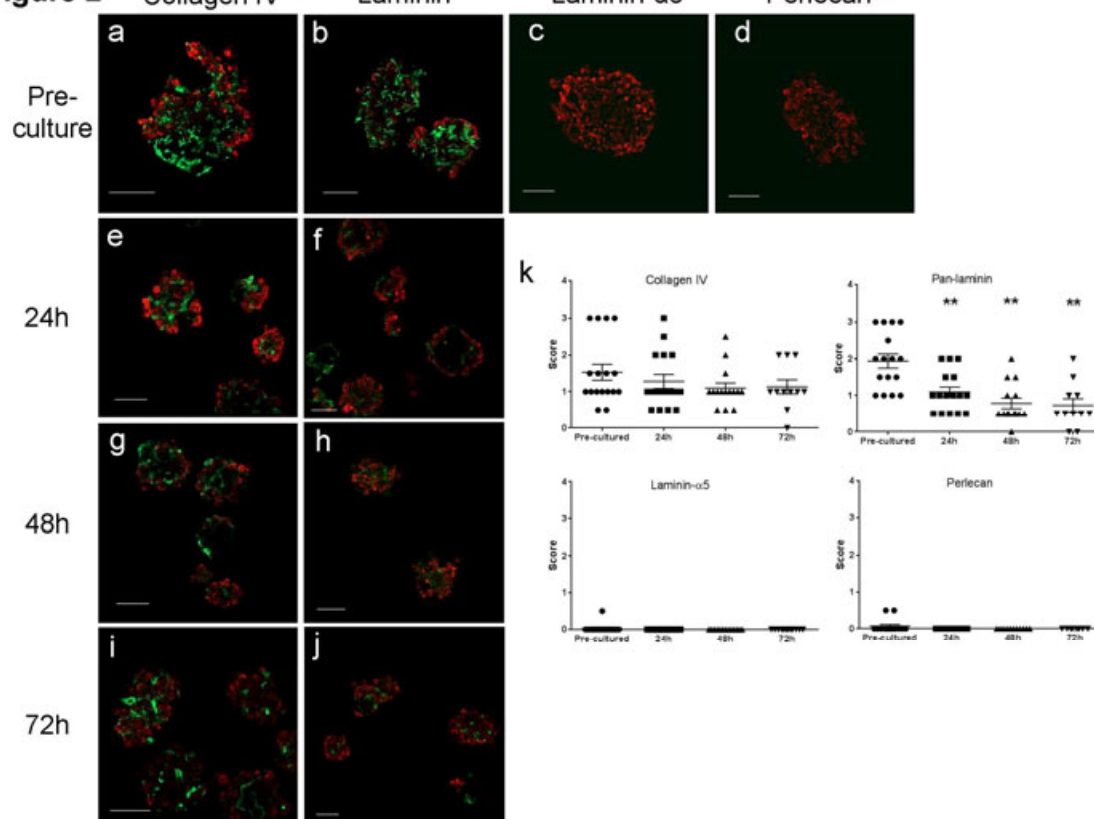
43	M	28	7	150	2	46	97	90	65	5
57	M	26	5	114	1	185	400	70	75	-
54	M	29	6	114	1	210	300	50	75	-
46	M	29	8.5	93	1	135	275	75	88	2.4
53	M	27	6.5	96	1	110	82	50	77	4
52	F	30	6	98	1	50	100	60	81	-
<b>51±1</b>	<b>13M</b> <b>14F</b>	<b>31±1</b>	<b>6.3±</b> <b>0.3</b>	<b>96±4</b>	<b>24 DBD</b> <b>3 DCD</b>	<b>118±19</b>	<b>209±18</b>	<b>61±3</b>	<b>77±2</b>	<b>4.0±0.8</b>

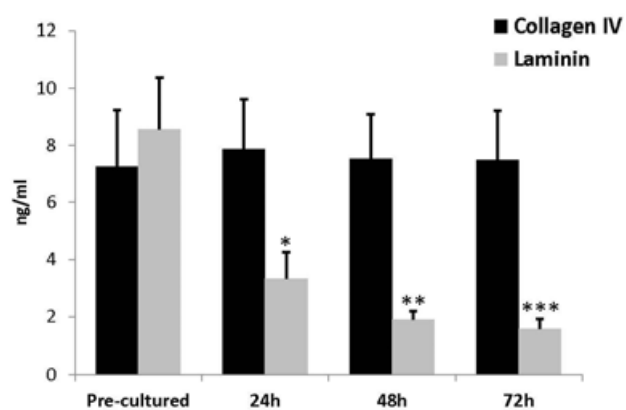
CIT, cold ischaemia time. IEQ, islet equivalent. SI, stimulation index (fold increase of insulin release from 1 to 20mM glucose). Data presented as mean±SEM. <sup>a</sup> Donor type: donation after brain death, DBD (1) or donation after cardiac death, DCD (2).

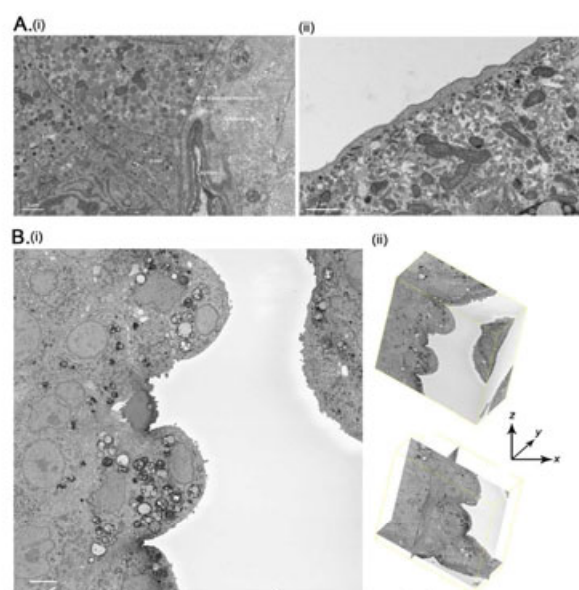
**Figure 1**

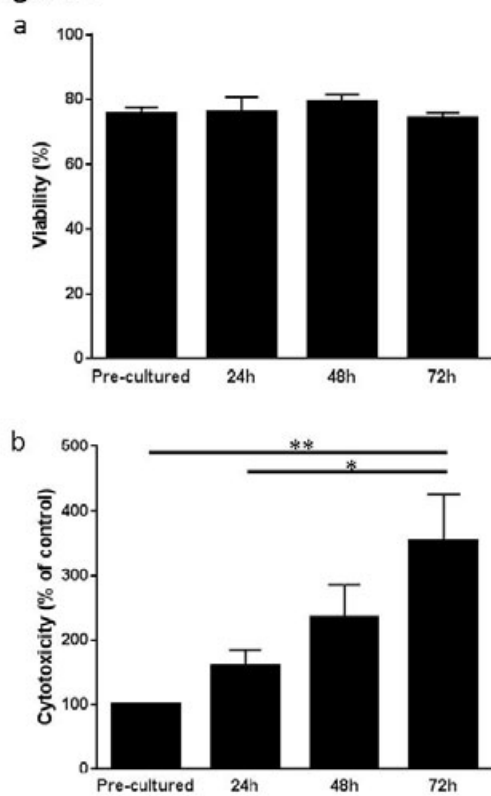


**Figure 2** Collagen IV Laminin Laminin- $\alpha 5$  Perlecan



**Figure 3**

**Figure 4**

**Figure 5**

**Figure 6**