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# IGF-2 coated porous collagen microwells for the culture of pancreatic islets†

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**Islet transplantation, the only curative therapy for type I diabetes, requires isolation of the graft in highly specialized facilities for its later dispatch to remote transplantation centres. During transport and culture, many valuable cells are lost due to several factors such as mechanical stress, islet aggregation and dissociation. Here, we evaluate a porous microwell array sheet made of natural collagen type I extracellular matrix (ECM) protein as a novel islet culture substrate. This culture platform can be coated with IGF-2, a growth factor favorable for islet survival, and allows segregation of the islets within the porous microwell sheet, preventing aggregation. This design shows promising results for improving human pancreatic islets viability and function during culture and could form a novel paradigm for the transport of islets between isolation and transplantation centres.**

## Introduction

Islets transplantation is, to date, the only available cure for type 1 diabetes. However, current transplantation protocols are hindered by the high loss of islet function that occurs during transport and holds prior to transplant. Up to 40% of islets are lost as they are moved from the isolation centre to the patient.[1] This cell loss is due to many parameters, such as apoptotic cell death triggered by hypoxic[2] and pro-inflammatory cytokine-mediated cell stress[3] or mechanical damage of the islets.[4] The result of this attrition is a reduction in the number of acceptable islet preparations for transplant, resulting in the need to transplant each patient with often two to three individual islet preparations. Consequently, islet transplantation is restricted to a small cohort of patients with severe conditions such as hypoglycemia unawareness.

Therefore, solutions that can enhance islet viability and function either during transport or culture after isolation have the potential to increase the number of islet preparations suitable for transplant, and thereby make the procedure available to more patients.

During current culture and transport conditions, islets can fuse together, resulting in an increase of the islet size, which increases the risk of hypoxia within the core of the islet mass and can lead to a significant loss of islet viability. Compartmenting the islets into microwells has the potential to reduce cell aggregation and possibly also mechanical stress. It has been shown that human islets separated on microwells exhibit a higher viability compare to planar surfaces.[5] However, microwells made of solid materials such as polystyrene or poly(dimethoxysilane) (PDMS) provide nutriment and gas permeation only from the top.[6,7]

We hypothesized that a scaffold with microwells that can reduce islet fusion, aggregation and mechanical stress while providing an anti-apoptotic growth factor would have the potential to improve islet viability during culture.

Porous collagen microwells (PCMs) are porous substrates made of type I collagen that are patterned by using an ice templating method.[8] It is a versatile, rapid and cost effective method to design scaffolds with complex architectures. Made of biocompatible collagen, such substrates are particularly interesting for their use in tissue engineering applications, cell culture or cell transport and implantation. Insulin-like growth factor – 2 (IGF-2) has been identified to have an anti-apoptotic effect on islet cells.[9] IGF-2 can be supplemented into the culture media of islet suspensions, but this strategy is limited by the half-life of the growth factors[10] and high doses of IGF-2 has been shown to be noxious for the islet cells.[11] In mammalian tissue, the extracellular matrix (ECM) acts as a reservoir for IGF-2, controlling its storage and release for interaction with the cells.[12]

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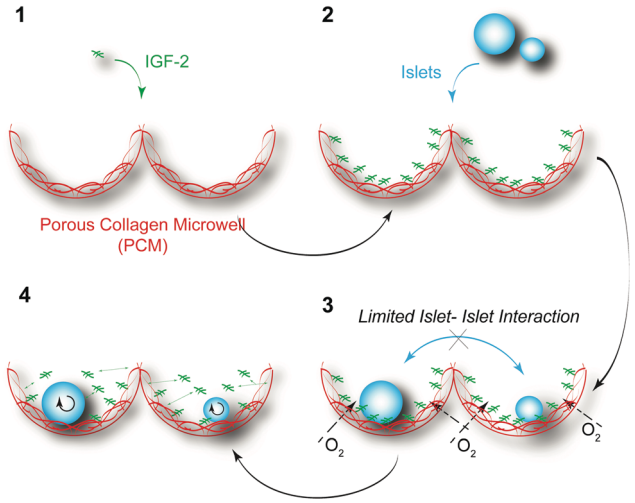


Scheme 1 Porous collagen microwell (PCM) arrays were coated with IGF-2, and then loaded with pancreatic islets. IGF-2 is allowed to interact with islets through contact with the immobilized molecules or desorption of the IGF-2.

Collagen, the most abundant ECM molecule in the human body, naturally binds to growth factors such as fibroblast growth factor 2 (FGF-2),[13] transforming growth factor beta 1 (TGF-β1)[14] and transforming growth factor beta 3 (TGF-β3).[15] Therefore, type I collagen can potentially serve as a materials platform to immobilize IGF-2 during islet culture.

In this study, we investigated the use of PCM arrays as three dimensional niches for islet cell culture. First, mouse islets were dispersed onto the PCM array on which IGF-2 was pre-adsorbed and islet viability on the IGF-2 coated PCM sheets were characterized. Then, these findings were confirmed with human islets by measuring adenosine triphosphate (ATP) levels and mRNA gene expression of lactate dehydrogenase in the islets cultured in PCM arrays compared to conventional islet culture following the current clinical isolation protocol (Scheme 1).

## Results and discussion

The PCM arrays were made by printing an array of water droplets on a hydrophobic surface. The droplets were frozen and collagen type I was cross-linked on top of the droplet array. After freeze drying of the scaffold, the collagen sheet possessed the imprints of the droplet array (ESI,† Fig. S1).[16] Materials and scaffolds intended for the transport of cells and especially for the interstate transport of islets need to be mechanically stable to withstand the stress encountered during shipping. Given that PCM arrays can tear and break upon stretching, the mechanical properties of the PCM arrays under tension and its tensile strength were characterized. We determined that the PCM array possessed a Young's modulus ($E$) of the foil of about 16.3 ± 0.6 kPa ($n$ = 3) and a tensile strength of 0.6 ± 0.3 N (ESI,† Fig. S2). This indicates that the PCM array is resilient under tension.

In addition to mechanical damage during isolation and transport, islets are susceptible to apoptosis during transport or during culture induced by the enzyme used during isolation, which can remain in the islet preparation.[17] Many recent studies have highlighted the benefits of IGF-2 for improving islet survival post-isolation,[18] as well as after transplantation.[19] Nevertheless, the amount of IGF-2 provided to the islets needs to be controlled, to avoid overdoses. One strategy to provide IGF-2 to islets during transport is to immobilize the molecule on the transport substrate. However, covalent immobilization does not reproduce the interaction between growth factors and natural components of the ECM. In contrast, adsorption of IGF-2 through inherent non-covalent interactions onto extracellular proteins (such as collagens) could deliver factors *via* simple re-solubilization.[20] While uniform growth factor adsorption can be easily achieved on planar surfaces,[21] this is more challenging in the three-dimensional porous structure of the PCM array. In order to offer maximum and homogeneous contact with the islet, the growth factors were presented at the bottom of the microwells (Fig. 1A) but also on the top of the array (Fig. 1B). Firstly, we validated that the IGF-2 was adsorbed onto the collagen. Biotinylated IGF-2 was reacted with FITC labelled avidin (1 : 1 ratio) to afford a fluorescent tracer of the growth factor. Incubation for 16 h, followed by successive rinsing with PBS,

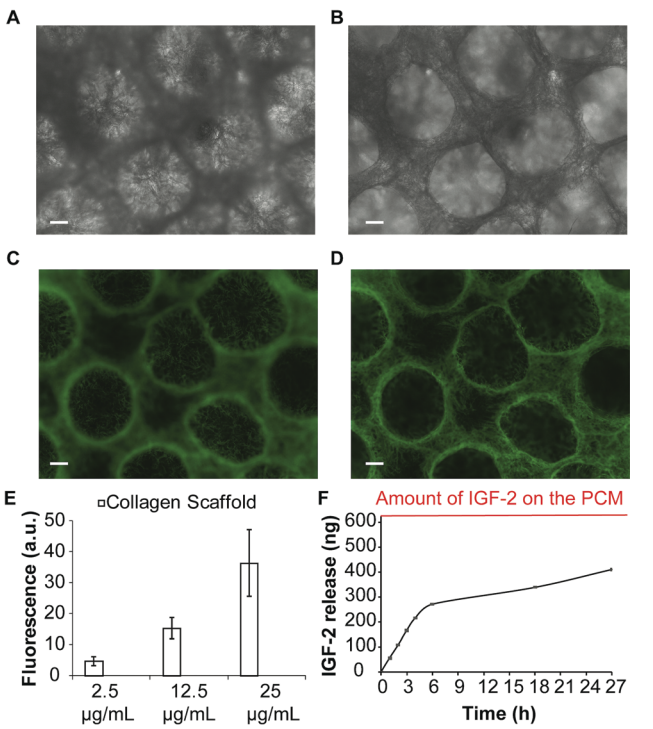


Fig. 1 Brightfield microscopy images of the PCM array focused on (A) the bottom and (B) the top of the microwells. Fluorescence microscopy images of the fluorescently labelled IGF-2 bound to collagen microwells, focused on the (C) bottom and (D) top of the microwells. Scale bars: 250 μm. (E) Semi-quantitative characterization of the amount of IGF-2 immobilized on the PCMs showing the increase IGF-2 immobilization on the PCM array with increase of the concentration in the coating solution showing that the loading of IGF-2 on to the PCM can be modulated. (F) IGF-2 released from the coated PCM array into PBS over time, as determined *via* fluorescent measurement, showing two domains of release over 24 h. Error bars are standard error for $n$ = 6 replicates.

revealed that the IGF-2 was uniformly immobilized on top of the PCM array, as well as within the microwells (Fig. 1C and D). In order to rule out any contribution to the fluorescence signals of unconjugated FITC labelled avidin, the PCM sheet was incubated with FITC-avidin alone, which showed negligible adsorption onto the PCM array (ESI,† Fig. S3A). Similarly, the auto-fluorescence of the collagen and the unlabelled IGF-2 was found to be negligible (ESI,† Fig. S3B). In order to determine if the loading of IGF-2 on the PCMs could be controlled through the concentration of the loading solution, we incubated the PCMs with FITC labelled IGF-2 solutions of 2.5, 12.5 and 25 μg $mL^{-1}$ and measured the fluorescence on the FITC labelled IGF-2 coated PCM arrays (Fig. 1E). This revealed an increasing amount of IGF-2 on the scaffold with increasing concentration of the IGF-2 in solution. The fluorescence of the IGF-2 solution in the supernatant after incubation with the PCM array showed that for an IGF-2 concentration of 12.5 μg $mL^{-1}$, the scaffold bound all of the growth factor, *i.e.* a negligible fluorescence signal was observed in the supernatant, (ESI,† Fig. S4). While the signal obtained for the PCM array incubated in the 25 μg $mL^{-1}$ solution is higher, some FITC labelled IGF-2 remained in the solution supernatant after incubation with the PCM, suggesting that not all of the IGF-2 was immobilized onto the PCM. Therefore, subsequent IGF-2 loaded arrays were prepared by incubation of the PCM arrays with 12.5 μg $mL^{-1}$ IGF-2 solutions, which allows for the efficient adsorption of the growth factor on the PCM. Since the IGF-2 is only physisorbed onto the PCM arrays, its release into the solution could be followed over time. PCM arrays were incubated for 16 h with 12.5 μg $mL^{-1}$ of FITC labelled IGF-2. Then, the IGF-2 coated PCM were immersed in PBS and the solution was changed 8 times over 24 h and the fluorescence of the incubated PBS solution was determined (Fig. 1F). The cumulative release showed that after a fast linear release over the first 6 h, the IGF-2 was then released at a slower rate for the next 18 h. This feature allows for the IGF-2 to be released from the PCM array and for controlled delivery of the active molecule to the islets during culture or transport.

During the transport of islets and manipulation during culture, movement of the container forces the islets to move and to come in contact with one another, inducing islet aggregation and dissociation, and further damaging the islets through shear stress.[22] We hypothesized that the use of PCM arrays would allow segregation of the islets and reduce interactions. In order to verify this, mouse islets were loaded on the PCM array and allowed to settle for 16 h. During this time, islets gravitated into the microwells of the PCM array (Fig. 2A and B). The efficiency of loading was characterized by manually counting the number of islet per microwells. We calculated that the bare and IGF-2 coated microwells were loaded with on average ~2 islets/well (Fig. 2C and D), although the distribution was skewed towards higher numbers for the IGF-2 coated microwells, no significant difference was observed ($p > 0.05$, *T*-test). It is known that islet can bind to IGF-2 *via* the IGF-2 membrane receptor.[23] Potential immobilization of the islet through IGF-2 receptor might also suppress islet movement over microwells. However, in both PCM and IGF-2 coated PCM, no single cells originating from islet dissociation, nor islet aggregation, was observed, in contrast to islets cultured on standard tissue culture polystyrene (TCPS) (ESI,† Fig. S5), suggesting that the microwells help prevent islet dissociation and aggregation.

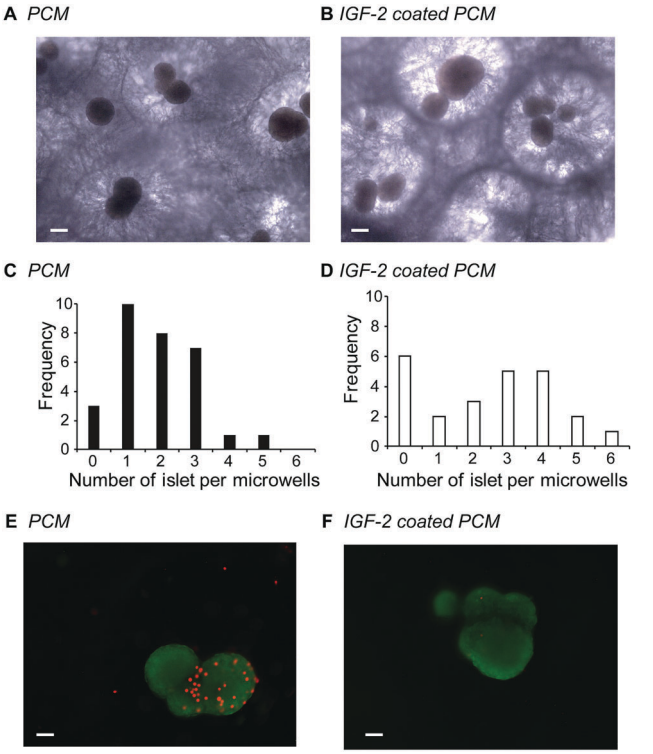


Fig. 2 Brightfield microscopic images of mouse islets within the uncoated PCM array (A) and IGF-2 coated PCM array (B) showing islet seggregation afforded by the microwells. Scale bar 100 μm. Distribution of the number of islets per microwells for the (C) uncoated and (D) for the IGF-2 coated PCM array, from 10 different areas of the PCM array. Fluorescence microscopy images of FDA/PI stained mouse islets loaded on (E) PCM and (F) IGF-2 coated PCM arrays, showing living cells (green) and dead cells (red). Scale bars: 200 μm.

In order to evaluate the ability of the PCM arrays coated with 0.25 μg $mL^{-1}$ IGF-2 to improve islet viability, mouse islets viability after 16 h culture in the PCM array was assessed using a live/dead assay. This revealed that fewer dead cells (red stained) were observed on the IGF-2 coated PCMs compared to the uncoated PCMs (Fig. 2A and B and ESI,† Fig. S6). These results suggest that the IGF-2 coated PCM can improve islet viability during culture.

In order to demonstrate that these findings translate to human cells, human islets obtained from a clinical isolation were cultured on an IGF-2 coated PCM. Human islets were cultured for 16 h on the uncoated PCM and coated PCM with IGF-2. The human islets were washed out from the array and stained with a live/dead assay (Fig. 3A and B). This staining shows that, similar to mouse islets, the human islets are viable on the IGF-2 coated PCM. However, the cell death observed for mouse islets on the uncoated PCM is not observed for human islets. Therefore, as an independent surrogate read-out of islet viability, we measured the ATP content of human islets cultured

 

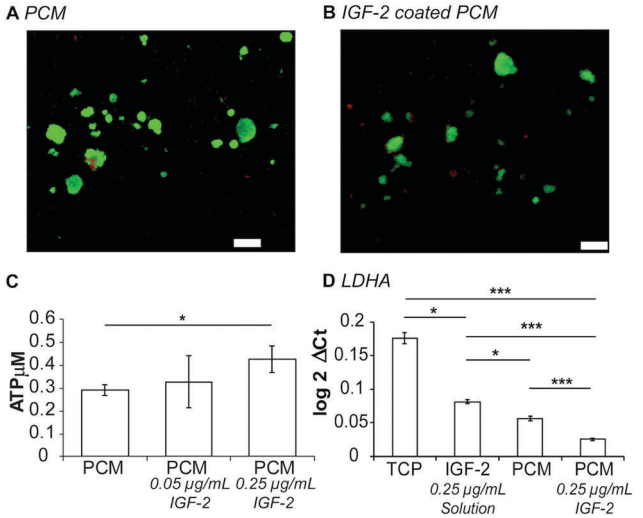


Fig. 3 Fluorescence microscopy images of FDA/PI stained human islets loaded on (A) PCM array and (B) IGF-2 coated PCM arrays, showing living cells (green) and dead cells (red). Scale bars: 200 µm. (C) ATP assay showing ATP content of human islets when cultured on IGF-2 coated PCM sheet. (D) LDHA gene expression, normalized to β-actin, for islets cultured on uncoated and IGF-2 coated PCM arrays, showing down-regulation of LDHA. Error bars represent standard error for $n = 3$. With * for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$, $t$-test two tails over technical triplicate.

in IGF2 coated PCM or uncoated PCM (Fig. 3C).[24,25] The ATP concentration was measured for the uncoated PCM arrays and PCM arrays coated with two different concentrations of IGF-2. Previously, we showed that the PCM is saturated with IGF-2 for solution with a IGF-2 concentration of 12.5 µg mL$^{-1}$. It has been demonstrated that a solution of IGF-2 at the concentration of 0.034 µg mL$^{-1}$ is beneficial for islet survival[26] but a higher concentration of 0.1 µg mL$^{-1}$ in solution can be detrimental to islet survival.[11] Therefore, we selected two concentrations below the apparent saturation level of the IGF-2 on the PCMs, *i.e.* lower than 12.5 µg mL$^{-1}$. We selected the 0.05 µg mL$^{-1}$ IGF-2 as a low level and 0.25 µg mL$^{-1}$ as a high level.

We observed that islets cultured on PCM with increasing IGF-2 adsorption onto the scaffold from 0.05 µg mL$^{-1}$ to 0.25 µg mL$^{-1}$ showed a slight increase of the ATP content as compared with islets cultured in the non-coated PCM. Human islets cultured conventionally on TCPS in medium supplemented with 0.25 µg mL$^{-1}$ of IGF-2 showed no significant difference in viability with unsupplemented controls (ESI,† Fig. S7). This result suggests that binding of IGF-2 onto the PCM array might be beneficial for the islet viability and function. Interaction between IGF-2 and the islets can occur through direct contact between the islets and the IGF-2 coated microwells or through growth factor release into the culture media over time (Fig. 1F). Islets consume a considerable amount of oxygen, which is proportional to their insulin production.[27] During culture, islets can suffer from hypoxia due to low oxygen circulation within the culture container or through aggregation, leading to higher cell death. Hypoxic stress can be indirectly measured by measuring the gene expression of lactate dehydrogenase A (LDHA). LDHA is overexpressed in stressed islets and has been shown to be associated with lower islet quality.[28] We compared the LDHA level of islets cultured in four different conditions: (i) conventional non-adherent tissue culture polystyrene (TCPS), (ii) non-adherent TCPS supplemented with 0.25 µg mL$^{-1}$ in the culture medium, (iii) islets seeded in the uncoated PCM array and (iv) in the 0.25 µg mL$^{-1}$ IGF-2 coated PCM array. It was observed that the level of LDHA in islets was downregulated when the culture media was supplemented with 0.25 µg mL$^{-1}$ of IGF-2, (Fig. 3D). This effect was further increased for islets cultured in uncoated PCM arrays and for islets cultured in IGF-2-coated PCM array. This might be due to the reduced islet aggregation provided by the microwell format, but also to the presence of adsorbed IGF-2, which can reduce islet stress during culture.

## Conclusions

Taken together, these results suggest a concomitant effect of the PCM array's microwell architecture, porous design and adsorbed IGF-2 on islet viability, function and stress. Admittedly, further studies need to be performed in order to clearly understand which mechanisms are responsible for the improvement in islets viability in this system. But our current study suggests that the described PCM format with immobilised IGF-2 has the potential to improve the survival of islets during culture and transport. This may pave the way towards the development of solutions to improve islet viability during their transport between isolation and transplantation centres.

## Experimental

### Methods

**Porous collagen microwells (PCM) fabrication.** The PCM sheet was prepared following a previously described procedure.[8] Pre-cooled collagen solution (1 wt%, Nippon Meat Packers, Inc. Osaka, Japan) was poured on the frozen ice template. The poured collagen solution and the ice template was frozen at −80 °C. The frozen collagen block with the ice templates was then detached from the PFA film-wrapped copper plate. The sample was then freeze-dried on a VirTis-Advantage Benchtop Freeze Dryer (SP Industries Inc., Warminster, USA) to form a porous collagen microwells sheet. This sheet was then cross-linked by glutaraldehyde vapor saturated with a 25% aqueous glutaraldehyde solution (GA, Wako, Osaka, Japan) in a closed box at 37 °C for 4 h. The PCM sheets were then washed with cold pure water, the unreacted aldehyde groups were block with an aqueous solution of 0.1 M glycine and further washed with cold pure water.

**Mechanical testing.** The PCM array with a thickness of 0.17 mm was cut into strip with a width of 8 mm and length of 40 mm. The samples were then positioned between the grips of a Shimadzu EZ-LX (Kyoto, Japan) mechanical tester. The elastic modulus ($E$) and force at breaking was measured by pre-loading the sample with a force of 0.1 N and subsequent testing at the rate of 1 N min$^{-1}$ until break. The breaking point was determined by a loss of force of 10% and the elastic modulus

was calculated by measuring the slope of the stress *versus* strain curve between 0.4 N and 0.8 N. All calculations were performed on the software provided with the instrument: Trapezium X from Shimadzu (Kyoto, Japan).

**Growth factor immobilization.** The PCM array was cut into the size of a 96 well with a biopsy punch of 6 mm diameter, and placed in the well plate. Samples were then soaked in 100 μL of PBS (pH: 7.8, 28 mM) for 1 h. Biotinylated IGF-2 from Gropep (Adelaide, Australia) was first solubilized in a 10 mM HCl solution then mixed 1 : 1 with the avidin-FITC from Sigma Aldrich (St. Louis, Missouri) solubilized in PBS and allowed to react for 1 h at 4 °C. The resulting FITC IGF-2 conjugate was diluted in PBS to obtain 2.5, 12.5 and 25 μg $mL^{-1}$ solutions of the growth factor. The PCM arrays were incubated with 200 μL of the IGF-2 solutions for 16 h at 4 °C and washed thoroughly three times with 50 μL PBS to remove the unbound growth factor. The supernatant and washing solutions retained for further analysis. The solution and the PCM arrays functionalized with IGF-2 were characterized on a plate reader Biotek Synergy HT (Winooski, Vermont) at the excitation wavelength of 485 nm and emission wavelength of 525 nm.

**IGF-2 release from PCM.** PCM disks of 6 mm diameter were prepared as described in the above section. The PCM was pre-soaked in PBS, then FITC labelled IGF-2 was added to obtain a final volume of 50 μL at a concentration of 12 μg $mL^{-1}$ (IGF-2 total amount 600 ng) and was incubated with the PCM for 16 h at 4 °C. Then, the IGF-2 coated PCM were incubated in 50 μL PBS for various time intervals and the fluorescence of the supernatant was measured on a plate reader. In order to correlate the fluorescence value to the IGF-2 concentration released out of the PCM a standard curve (ESI,† Fig. S8) was plot. Solutions of IGF-2 at concentrations ranging from 3 to 50 μg $mL^{-1}$ were prepared as six replicates and the fluorescence was measured on a plate reader.

**Mouse islet isolation and culture.** Mouse islets of Langerhans were isolated using collagenase P (Roche, Basel, Switzerland) and Histopaque-1077 density gradients (Sigma-Aldrich) as previously described.[29]

**Human islet isolation and culture.** Human islets were obtained through the isolation centre at the St Vincent's Institute (Melbourne, Australia), with appropriate human ethics approval. St Vincent's Hospital (Melbourne, Australia) Human Research Ethics Committee Protocol Number: HREC-011-04.

**Mouse and human islet culture in the PCM.** The scaffolds were pre-incubated with PBS or IGF-2 at the indicated concentration for 16 h and in case of IGF-2, were rinsed three times before use. The PCM arrays were transferred into a fresh 96 well plate and 20 handpicked islets were loaded per PCM insert in complete Connaught Medical Research Laboratories (CMRL) medium were seeded for 16 h. Pictures of islets were acquired on an upright non-inverted microscope Leica DFC295 (Wetzlar, Germany) at 4× magnification. The number of islet per microwell was then counted manually and the distribution was calculated in Microsoft Excel (Redmond, Washington).

**Live/dead assay.** After 24 h in culture, arrays containing islets were carefully removed from the well plates, and islets within the array were stained by adding 2.5 μg $mL^{-1}$ fluorescein diacetate (FDA) and 10 μg $mL^{-1}$ propidium iodide (PI) from Sigma Aldrich (St. Louis, Missouri) in Roswell Park Memorial Institute (RPMI) medium for 5 min in the dark. Islets were imaged using the non-inverted Leica DFC295 microscope at 10× magnification.

**ATP assay.** Islets were cultured for 24 h in the PCM arrays with or without IGF-2 coatings, and the ATP content of 20 islets per array was analyzed using CellTiter-Glo3D Cell Viability Assay from Promega (Madison, Wisconsin, USA) and ATP standard curve method according to the protocol provided.

**RT-PCR.** Extraction and purification of RNA was performed using an Ambion RNAqueous Micro Kit from Thermo Fischer Scientific (Waltham, Massachusetts) according to the manufacturer's instructions. The purified RNA was quantified by absorbance at 260 nm using a Thermo Fischer Scientific Nanodrop 2000. 200 μg of RNA was reversed transcribed in a 20 μL reaction using Biorad (Hercules, California) iScript Reverse Transcription Supermix for RT-qPCR, according to the manufacturer's instructions. The complete reaction mix was diluted 1 : 2 following an incubation of 5 min at 25 °C, 30 min at 42 °C and 5 min at 85 °C. Real-time quantitative PCR analysis was performed on triplicate samples, using 2 μL of cDNA using the following gene-specific Taqman® primer from Thermo Fischer Scientific: LDHA (Mm01612132_g1). B-actin (ACTB Mm02619580_g1) was used as a housekeeping gene and to normalise expression data using log 2 (Δct) as a method for quantitation.

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