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Effect of alginate encapsulation on the cellular transcriptome of human islets

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ABSTRACT

Encapsulation of human islets may prevent their immune rejection when transplanted into diabetic recipients. To assist in understanding why clinical outcomes with encapsulated islets were not ideal, we examined the effect of encapsulation on their global gene (mRNA) and selected miRNAs (non-coding (nc) RNA) expression. For functional studies, encapsulated islets were transplanted into peritoneal cavity of diabetic NOD-SCID mice. Genomics analysis and transplantation studies demonstrate that islet origin and isolation centres are a major source of variation in islet quality. In contrast, tissue culture and the encapsulation process had only a minimal effect, and did not affect islet viability. Microarray analysis showed that as few as 29 genes were up-regulated and 2 genes down-regulated (cut-off threshold 0.1) by encapsulation. Ingenuity analysis showed that up-regulated genes were involved mostly in inflammation, especially chemotaxis, and vascularisation. However, protein expression of these factors was not altered by encapsulation, raising doubts about the biosignificance of the gene changes. Encapsulation had no effect on levels of islet miRNAs. *In vivo* studies indicate differences among the centres in the quality of the islets isolated. We conclude that microencapsulation of human islets with barium alginate has little effect on their transcriptome.

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1. Introduction

The advent of a steroid-free immunosuppressive protocol made human islets transplantation a promising therapy for patients with type 1 diabetes [1,2]. However, a major limiting factor is chronic immunosuppression with drugs, which increase the risk of infection and neoplasia and can cause organ toxicity. Microencapsulating human islets is a strategy that could prevent rejection of the grafted tissue without the need for anti-rejection drugs. Alginate hydrogel is the most popular polymer used in generation of islet microcapsules [3]. Allografts and in some cases, xenografts function without the need for anti-rejection drugs, at least in rodents, with recipients becoming normoglycaemic (reviewed in [4]). In humans, such success has yet to be achieved, although some graft function is observed [5,6]. Although reasons for this are unknown, islet hypoxia and inflammatory responses are believed to be the major responsible factors [7].

When encapsulated, insulin-producing cells continue to function and secrete insulin. The diameter of the capsule can affect cell viability, especially if the size is large, thereby limiting passage of oxygen and other nutrients to the encapsulated cells [8–10]. As well, the alginate composition and encapsulation can affect the level of insulin secreted, which can be attributed to the interactions between guluronic/mannuronic polysaccharides and divalent cations and reduced TCA cycle activity [11]. Whether alginate encapsulation also affects the transcriptome of the β cell is unknown. Here, we report global gene expression analysis and microRNA (miRNA) profiling of human cadaveric islet preparations obtained from three islet isolation centres.

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2. Materials and methods

2.1. Human islet isolation and shipment

Cadaveric human pancreases were obtained from brain-dead donors with informed consent of their relatives. For this study human islet from three different islet isolations centres (designated as 1, 2 and 3) were used. In all the centres, human islets were isolated using a modification of the method described by Ricordi et al. [12,13]. Details of the procedure at each of these centres can be found in Table 1. Isolated human islets were cultured in supplemented CMRL-1066 media containing 1.5% human albumin, glutamax, sodium pyruvate, insulin-transferrin selenium (ITS), nicotinamide and antibiotics (Mediatech, Herndon, VA) or Miami transplantation media before being shipped to Sydney using a commercial courier service. The islets were then cultured for a day before encapsulation. All the procedures regarding obtaining human islets were approved by the Institution's Human Research Ethics Committee. Dithizone staining was used to assess the presence of endocrine cells and purity of islet preparations.

2.2. Encapsulation

The cultured human islets were pooled together and washed in 0.9% NaCl before being suspended in highly purified 2.2% alginate (60:40 guluronic acid: mannuronic acid, UPMVG PRONOVA, FMC Biopolymer) solution in 1:8 ratio. The microcapsule formation was carried out with an air-driven droplet generator (Steinau, Berlin, Germany) as described previously [14]. The microcapsules formed were incubated in a bath of 20 mM barium chloride for 2 min. After gelation, the microcapsules were washed thrice in 0.9% NaCl to remove excess barium. The average size of the microcapsules was 560 μm (range 500–700 μm, median – 550 μm).

2.3. Tissue culture

For all the *in vitro* studies, 1000–3000 IEQs of non-encapsulated and encapsulated human islets were cultured in CMRL-1066 media for 1, 3 and 7 days respectively. For the *in vivo* studies 3000, 2000 and 1000 IEQs of encapsulated human islets were cultured for 24 h and subsequently transplanted the following day into the peritoneal cavity of diabetic NOD/SCID (Non-obese Diabetic/Severe Combined Immunodeficient) mice.

2.4. Viability

Viability of both non-encapsulated and encapsulated human islets was assessed using the fluorescent dyes 6-carboxyfluorescein diacetate (6-CFDA; Sigma, St.Louis, MO) and propidium iodide (PI; Sigma). The percentage of green (live cells) to red (dead cells) fluorescence was assessed to evaluate the viability ($n = 100$, for each time point and preparation). Samples were analyzed with a Zeiss-Axioskop 2 microscope using Axiovision LE software.

2.5. Insulin secretion and content

Aliquots of non-encapsulated and encapsulated human islets from each isolation centre (20 islets, for each preparation) were exposed to either 2.8 mM glucose (basal, $n = 3$) or 20 mM glucose (stimulus, $n = 3$) for 1 h at 37 °C with gentle agitation. The supernatant was then collected and human insulin measured by radioimmunoassay (RIA; Linco, St Charles, MO, USA). The remaining pellet was washed in Hanks Balanced Salt solution (HBSS) solution followed by addition of cold acid ethanol and vortexed vigorously to enhance cell lysis. The cell extract was kept at 4 °C overnight and the supernatant collected the following day for measuring insulin content of the cells by RIA.

2.6. Decapsulation

The encapsulated human islets were washed twice in PBS and 15 ml of decapsulating solution (50 mM EDTA and 10 mM HEPES in PBS) was added and incubated for 15 min at 37 °C. Once the alginate was dissolved, the cell suspension was transferred to a 50 ml falcon tubes and mixed several times to break the cell clusters. The cell pellet was obtained by centrifugation at 3000 rpm for 10 min and subsequent removal of the supernatant. The decapsulated cells were then used for total RNA extraction either through Trizol or RNAspin Mini kits.

2.7. Affymetrix microarray analysis

For the microarray experiments, 3000 IEQs of non-encapsulated and encapsulated human islets from each of the three centres were cultured for 1, 3 and 7 days respectively. At each time point, the encapsulated islets were decapsulated and the total RNA extracted. Total RNA was extracted using Trizol reagent (Invitrogen, Burlington, Canada), purified by RNeasy columns (Qiagen, Hilden, Germany) and the quality assessed by agarose gel electrophoresis and capillary electrophoresis (Agilent 2100 Bioanalyser; Agilent, Palo Alto, CA, USA). The gel picture showed the presence of distinct 28S and 18S ribosomal RNA bands (Supplementary Figure 1).

Table 1
Variables associated with islets isolated from the three isolation centres. All pancreases were maintained in UW solution prior to islet isolation. Values = mean ± SEM.

Islet isolation Centre	Donor age (yr)	Gender (male/female)	BMI (kg/m^2)	Pancreas weight (g)	Appearance of pancreas	Enzyme blend	Cold ischemic time (h)	Purity (%)	SI (pre-encapsulation)	Viability (pre-encapsulation) (%)	SI (post-encapsulation)	Viability (post-encapsulation) (%)
1 ($n = 4$)	30.2 ± 2.7	M (40%) F (60%)	29.6 ± 1.6	74.7 ± 4.4	I – 100% HF – 40% M/NF – 60%	Liberase HI (Roche)	8.1 ± 0.6	85.6 ± 3.5	3.9 ± 0.7	80.5 ± 2.5	4.1 ± 0.6	77.5 ± 2.5
2 ($n = 6$)	35.6 ± 4.8	M (63%) F (38%)	25.3 ± 2.1	72.1 ± 6.4	I – 54% NI – 46% HF – 46% M/NF – 54%	Collagenase NB (Serva)	7.4 ± 0.6	80.7 ± 3.4	2.6 ± 0.4	75.5 ± 4.1	3.2 ± 0.4	70.5 ± 3.3
3 ($n = 3$)	50.2 ± 5.5[a]	M (60%) F (40%)	23.8 ± 0.6	70.7 ± 5.1	I – 100% HF – 60% M/NF – 40%	Liberase HI (Roche)	6.9 ± 1.2	70.1 ± 2.1[b]	1.9 ± 0.4[b]	76.5 ± 6.5	2.4 ± 0.3[b]	70 ± 5

[a] Centre 3 > Centres 1 and 2.
[b] Centre 3 < Centres 1 and 2 (ANOVA and Duncan's multiple comparison test). I, intact, HF, high fat, M/NF, moderate/no fat, SI, stimulation index, BMI, body mass index.

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The yield of total RNA extracted was between 140 and 625 ng/μl and the 260 nm/280 nm ratio of spectrophotometry reading was approximately 2.0. A total of 250 ng of RNA was reverse transcribed into cRNA and biotin-UTP labeled using the Illumina TotalPrep RNA Amplification Kit (Ambion). cRNA was quantified using a nanodrop spectrophotometer and the cRNA quality (size distribution) was further analyzed on a 1% agarose gel. cRNA was hybridized overnight to the Illumina HumanWG-6 Expression BeadChip, washed and stained using standard protocols (provided by Illumina). The arrays were scanned using an Illumina BeadArray Reader. Following average normalization, Principal component analysis (PCA) was undertaken to elucidate the possible sources of variation (Partek software) and differentially expressed genes were displayed in the Volcano plot format. Multiple testing correction was performed, and a false-discovery rate of 10% was set as threshold.

Data were normalized using "average" normalization in Beadstudio®. Comparative data analysis was performed using the Partek® Genomics suite software, including principal component analysis (PCA) as a means to visualize sources of variation. Differentially expressed genes between individual conditions were extracted based on false-discovery rate (FDR) calculations with a threshold set at 10% FDR, and projected using volcano plots in selected dimensions (Partek® Genomics suite software). Batch effect normalization was applied in Partek® to re-normalize data hereby reducing variation occurring from the measurement process.

2.8. MicroRNA analysis

For the microRNA expression analysis, 2000 IEQs of non-encapsulated and encapsulated human islets from each of the three centres were cultured for 1, 3 and 7 days respectively. At each time point, the encapsulated islets were decapsulated and the total RNA was extracted using Trizol reagent (Invitrogen, Burlington, Canada). miRNAs were assessed using a protocol described earlier [15]. Briefly, RNA was reverse transcribed using mature miRNA-specific primer sets (Applied Biosystems, Foster City, CA) and microRNA reverse transcription kit (Applied Biosystems, Foster City, CA). This was followed by real-time PCR (Q-PCR) using mature miRNA-specific TaqMan-based probe-primer sets (Applied Biosystems, Foster City, CA). The real-time PCR was carried out using 7500 FAST system (Applied Biosystems, Foster City, CA).

2.9. Real-time PCR

A total of 2000 IEQs of non-encapsulated and encapsulated human islets were cultured under normoxic conditions for 1, 3 and 7 days, respectively. At each time point, the encapsulated islets were decapsulated and total RNA was extracted from both non-encapsulated and encapsulated islets using the RNeasy mini kits (Qiagen, Hilden, Germany). The cDNA was prepared using the SuperScript III First-Strand Synthesis System and random hexamers (Invitrogen Corporation). Gene expression was determined by real-time PCR as described previously [16]. Briefly, for each reaction 2 μl of diluted cDNA, 10 μl of SYBR green master mix, 0.15 μl of 10 μM forward and reverse primers and 7.7 μl of nuclease-free water were used making a total volume of 20 μl. Q-PCR was carried out using the Mx3500P Real-time PCR system (Stratagene, NSW, Australia). The relative expression levels of the genes of interest were calculated using a mathematical model [17] based on the individual Q-PCR primer efficiencies and the quantified values were normalized against the housekeeping gene 18S. The primer sequences were:

18S: 5′-GTTCCGACCATAAACGATGC-3′ (forward),
5′-AACCAGACAAATCGCTCCAC-3′ (reverse),
MMP-9: 5′-TTGACAGCGACAAGAAGTGG-3′ (forward)
5′-GCCATTCACGTCGTCCTTAT-3′ (reverse),
CCL20: 5′-CCAAGAGTTTGCTCCTGGCT-3′ (forward),
5′-TGCTTGCTGCTTCTGATTCG-3′ (reverse),
CXCL6: 5′-GTTTACGCGTTACGCTGAGAGTAAA-3′ (forward),
5′-CGTTCTTCAGGGAGGCTACCA-3′ (reverse),
BMP4: 5′-CACAGCACTGGTCTTGAGTATCCTG-3′ (forward),
5′-CTCAGGGATGCTGCTGAGGTTAAAG-3′ (reverse),
CYR61: 5′-GTCACCCTTCTCCACTTGAC-3′ (forward),
5′-TGGGGACACAGAGGAATGCA-3′ (reverse),
CFB: 5′-GGAAGGGAATGTGACCAGG-3′ (forward),
5′-AAGGCAGGAGAGAAGCTGG-3′ (reverse),
HIF-1α: 5′-TCCAGTTACGTTCCTTCGATCA-3′ (forward),
5′-TTTGAGGACTTGCGCTTTCA-3′ (reverse).

2.10. Enzyme linked immunosorbent assay (ELISA)

A total of 1000 IEQs of non-encapsulated and encapsulated human islets were cultured for 1, 3 and 7 days respectively. MCP-1 (Monocyte chemoattractant protein − 1) ELISA was performed on culture supernatant in duplicates using a Quantikine MCP-1 ELSIA kit (R&D systems, USA) according to the manufacturer's instructions. Briefly, 200 μl of the culture supernatants was added to the wells and incubated for 2 h at 37 °C. The plate was washed with 400 μl wash buffer twice and 200 μl MCP-1 conjugate added followed by incubation for 2 h. After the addition of substrate and stop solution, the optical density was determined using a microplate reader (Bio-Rad 680 XR, Australia) at 450 nm with wavelength correction of 570 nm.

2.11. Western blots

A total of 2000 IEQs of non-encapsulated and encapsulated human islets isolated from different centres were cultured for 1, 3 and 7 days respectively. The encapsulated human islets were decapsulated and the human islets lysed using the cold lysis buffer (10 mM Tris, 10 mM NaH_2PO4/Na_2HPO4, 130 mM NaCl, 1% Triton-X100, and 10 mM sodium pyrophosphate) supplemented with protease inhibitors. The protein concentrations were determined using the Bradford method (Bio-Rad, Alfred Nobel Drive, Hercules, CA). Next, equal amount of protein were loaded on 10% Ready Gel TrisHCl gels (Bio-Rad, Australia), separated and transferred to a nitrocellulose membrane (Bio-Rad, Australia). The membrane was blocked for 1 h and then incubated with primary antibodies overnight at 4 °C. The membranes were subsequently washed with Tris-buffered saline containing tween [10 mmol/l Tris, 140 mmol/l NaCl, 0.02% Tween 20 (pH 7.6)] and probed with corresponding secondary antibody (1:500) for 1 h at room temperature. Proteins were detected using an ECL Plus western blotting detection system (Amersham Biosciences, Piscataway, NJ). The primary antibodies used in this study were rabbit anti-MMP-9 (Matrix metalloproteinase-9), mouse anti-BMP4 (Bone morphogenetic protein 4), rabbit anti-CYR61 (cysteine-rich protein 61), rabbit anti- HIF-1α (Hypoxia inducible factor-1α) (Novus biologicals USA), mouse anti-CFB (Complement factor B), mouse anti-CCL20 (Chemokine C-C motif ligand 20), rabbit anti-CXCL6 (Chemokine C-X-C motif ligand 6) (Abnova, USA) and rabbit anti-human actin (Sigma Aldrich, St.Louis, USA). All the primary antibodies except for actin (1:5000) were used at a dilution of 1:1000. The secondary antibodies were polyclonal goat anti-rabbit Ig/HRP and polyclonal goat anti-mouse Ig/HRP (1:5000, Dako).

2.12. Transplantation of microencapsulated human islets

Female NOD/SCID mice (6–8 weeks) were made diabetic by three consecutive intraperitoneal injections of streptozotocin (70 mg/kg) (Alexis Biochemicals). Animals with three consecutive blood glucose levels (BGLs) >12 mmol/L were considered diabetic and used for the study. Briefly, the mice were anaesthetized with pentobarbitone (65 mg/kg) and the encapsulated human islets were injected into the peritoneal cavity using a 20-gauge catheter. Mice were divided into three groups and each group was transplanted with 3000, 2000 or 1000 IEQs of encapsulated human islets isolated from each of the isolation centres. All the procedures involving the mice were approved by the relevant Animal Care and Ethics Committee.

2.13. Oral glucose tolerance test

Animals were considered normoglycemic if three consecutive random BGLs were <6 mmol/L. Once normoglycemia was achieved, an oral glucose tolerance test (OGTT) was performed. For this the mice were fasted overnight followed by an oral glucose gavage (3 mg/g of 300 mg/mL glucose solution) and BGLs were measured at 0, 20, 40, 60 and 120 min. OGTTs also were carried out in diabetic and non-diabetic control NOD/SCID mice.

2.14. Statistical analysis

All values were expressed as mean ± SEM. The statistical software NCSS97 was used to perform the analysis of data. One-way analysis of variance and Duncan's multiple comparison tests were used to compare data among groups, and Student's *t*-test between the groups. The results were considered significant when *P* values were <0.05.

3. Results

3.1. Assessment of the human islets from three isolation centres

As seen in Table 1, islets isolated in Centre 3 were mostly from older donor pancreata, compared to Centres 1 and 2. Centres 1 and 3 used Liberase HI as the digestive enzyme compared to Centre 2 which used Collagenase NB. Dithizone staining showed that the islets obtained from Centres 1 and 2 were highly pure (>80%) compared to Centre 3. The islets isolated from all the three centres had a viability of >75% pre-encapsulation. Post-encapsulation, there was a decline in viability, but this was not statistically significant. There was also no significant difference in other parameters amongst the three centres, including body mass index, preservation solution used, weight of the pancreas and cold

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ischemic time. Non-encapsulated and encapsulated islets from all the preparations responded to glucose with stimulation index ranging from 1.9 to 4.1; with islets from Centre 3 having the lowest stimulation index.

3.2. Global gene expression analysis

3.2.1. PCA analysis

A total of 12 scans were obtained and these covered human islets preparations from the three different isolation centres with samples analyzed prior to and following encapsulation. PCA mapping of the microarray data on the samples obtained from non-encapsulated and encapsulated islets that were cultured for 1, 3 and 7 days showed overlapping of the clusters, hence dismissing culture time points as a major source of variation (Fig. 1A). On the other hand, PCA analysis of non-encapsulated islets from the different isolation centres showed apparent segregation, defining a clear difference in the origin of the preparation (Fig. 1B). Further, comparing the origin of islet preparations in three dimensions (Supplementary Figure 2) showed significant changes in the gene expression suggesting all islet preparations were intrinsically different.

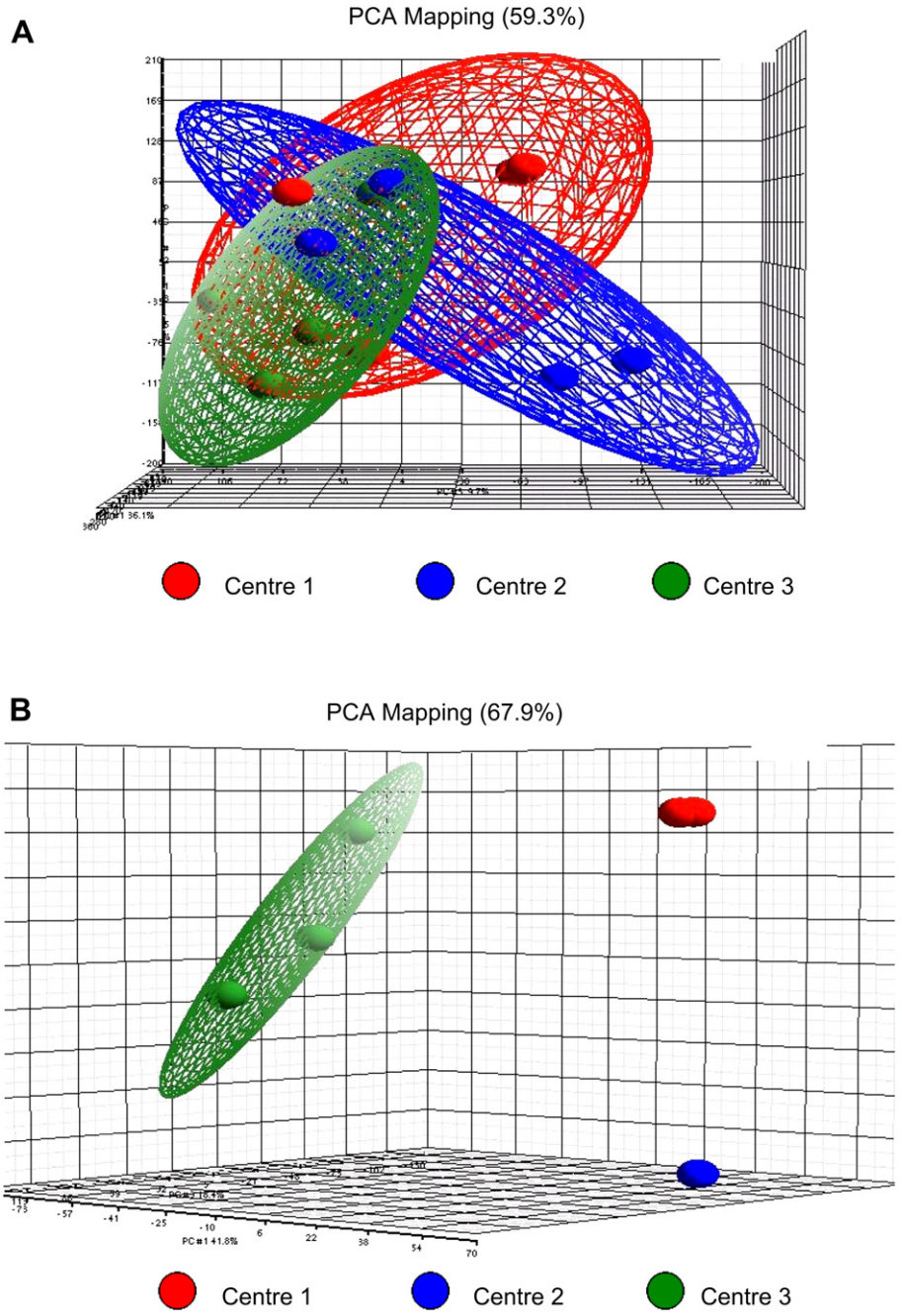

Fig. 1. PCA analysis was carried out to elucidate the two possible sources of variations namely (A) the culture time points and (B) isolation centre effect. (A) Non-encapsulated and encapsulated human islets were cultured for 1, 3 and 7 days respectively. PCA analysis with orthogonal projection of the data with ellipsoids drawn around group of samples cultured for 1,3 and 7 days, showed overlapping of the clusters; hence dismissing culture time points as a major source of variation. (B) Non-encapsulated islets from different centres were cultured for 1 day. PCA analysis showed that there was a clear separation among islets isolated from the different centres suggesting the "Centre effect" as a possible source of variation.

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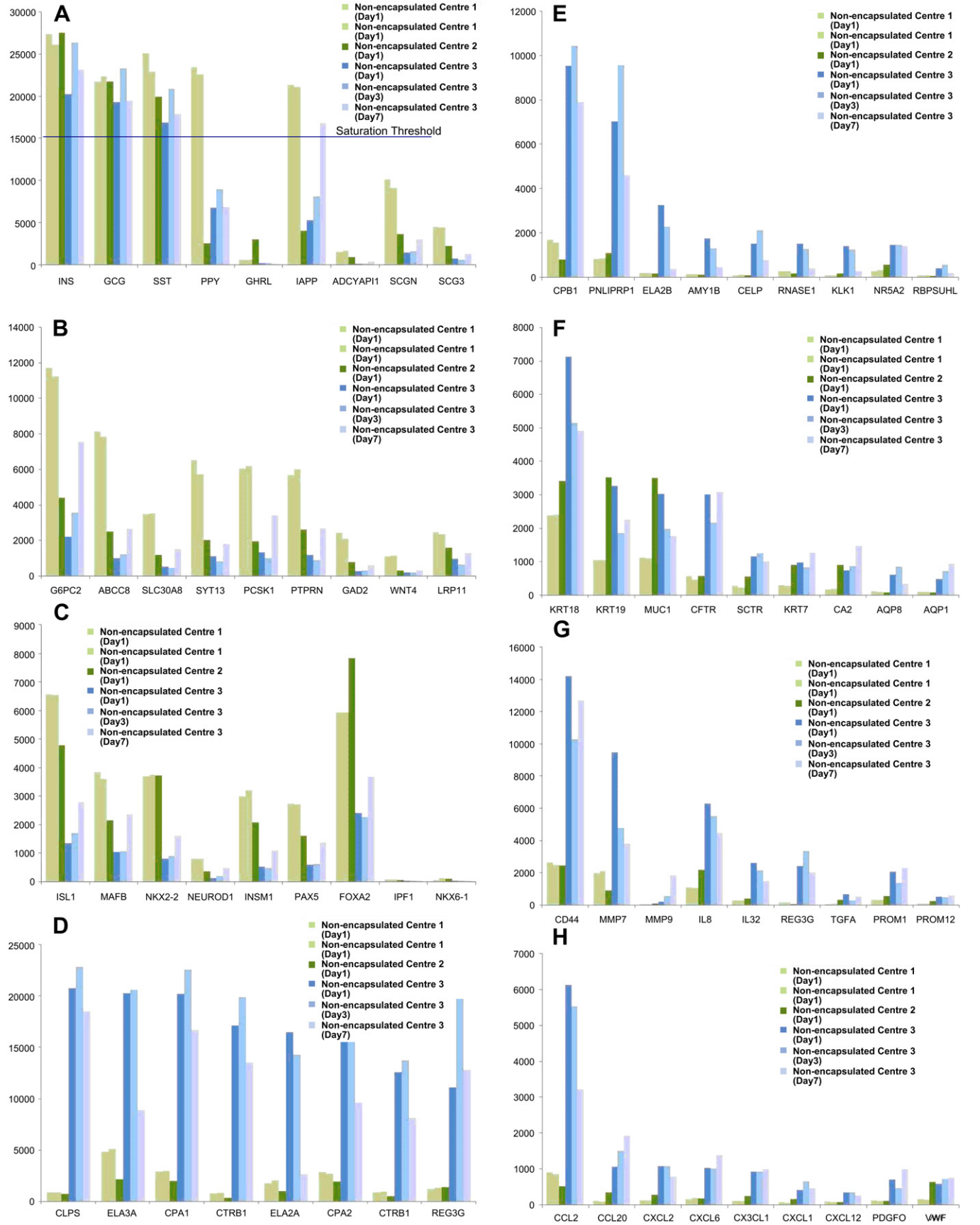

Fig. 2. Expression of endocrine, exocrine, ductal, regeneration associated and chemokine/growth factor related genes in non-encapsulated islets isolated from different centres. The normalized mRNA expression levels of (A) endocrine terminal products, (B) islet-specific expression and (C) islet-expressed transcription factor encoding genes in islets isolated from three isolation Centres 1, 2 and 3. All the endocrine related genes were highly expressed in islets from Centre 1 compared to Centres 2 and 3. The exocrine expressed (D and E) and ductal related genes (F) were highly expressed in islets from Centre 3 compared to Centres 1 and 2. Similarly, the normalized mRNA expression levels of (G) regeneration associated and (H) chemokine genes were highly expressed in islets from Centre 3 compared to Centres 1 and 2.

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3.2.2. Non-encapsulated human islets

Insulin was the most abundantly expressed endocrine gene, but the signal intensities for this and that for glucagon and somatostatin were at saturation threshold in islet preparations from all centres, thereby preventing determination of any differences among the centres (Fig. 2A). Other endocrine hormones, secretory granule markers and islet transcription factors were expressed most highly in the islets isolated at Centre 1 (Fig. 2B and C). In contrast, exocrine genes were most highly expressed in the islet preparations obtained from Centre 3, with CLPS, CPA1, CPA2, CTRB1, CELP, REG3G and elastase (ELA2A, ELA3A) having a signal density similar to that of insulin in that centre (Fig. 2D and E). Gene expression data correlated with the assessment of islet purity (Table 1) confirming that islets from Centre 3 were least pure. As might be expected, ductal specific genes were more highly expressed in preparations from this centre, but also Centre 2 (Fig. 2F). The regeneration associated genes, especially matrix metalloproteinase, interleukins, chemokines, such as CCL2/MCP1 and growth factor genes, such as PDGFD and VWF, were most highly expressed in islets from Centre 3 (Fig. 2G and H).

3.3. Encapsulated human islets

Comparing the individual preparation of the same origin to the result of this sample undergoing encapsulation (non-encapsulated day 1 vs encapsulated day 7) showed very few changes in the gene expression profile, as can be seen in volcano plots (Supplementary Figure 3). These plots were the results of a differential expression analysis, using a false-discovery cut-off threshold p value of 0.1. As few as 29 genes were up-regulated in all islet preparations as a result of encapsulation and 2 genes were down-regulated (Fig. 3 and Supplementary Table 1). The biological effects of the 29 up-regulated genes, analyzed with Ingenuity Pathways Analysis (Ingenuity Systems R at www.ingenuity.com), were mostly in enhancing inflammation, especially by chemotaxis, and vascularisation. Details of the seven biofunctional groups identified were described in Supplementary Table 2. Importantly, genes were identified neither for inflammatory cytokines nor hypoxia.

3.4. Real-time PCR analysis and Western Blots

To substantiate the results obtained by differential expression analysis, six common genes identified as having some biological relevance were selected and analyzed by real-time PCR. The genes were MMP-9, CXCL6, CCL20, BMP4, CFB, CYR61 and HIF-1α. There was no significant difference in the gene expression levels comparing non-encapsulated islets with the corresponding encapsulated islets cultured for days 1, 3 or 7. However, the gene expression levels increased with duration of culture of both non-encapsulated and encapsulated islets (Fig. 4A). However, neither duration of culture nor encapsulation had an effect on protein expression of these agents (Fig. 4B i and ii).

3.5. MCP-1 expression levels

Chemokines are associated with beta cell destruction, and their blockade has been shown to prolong islet allograft survival [18,19]. Further, it has been demonstrated that islets with low levels of MCP-1 function better than those with higher levels of this chemokine [20,21]. In our study, analysis was carried out to determine whether encapsulation cause an increase in the MCP-1 levels secreted by human islets and its effects on transplantation outcomes. To do this, 1000 IEQs of both non-encapsulated and encapsulated islets were cultured for 1, 3 and 7 days and the culture supernatants collected. The secretion of MCP-1 varied between different preparations and had a wide range of inter- and intra- islet variabilities. Highest levels of MCP-1 protein were detected in the culture supernatants from Centre 3 for both non-encapsulated and encapsulated islets (Fig. 5). However, the levels of MCP-1 in the encapsulated islets were similar to those in the corresponding non-encapsulated islets, indicating that encapsulation had no effect on MCP-1 levels secreted by human islets (Fig. 5).

3.6. MicroRNA analysis

To investigate whether encapsulation affects the miRNA expression profile, we looked at the expression of islet-specific miRNAs, miRNA-7, miRNA-9, miRNA-29b, miRNA-124a, miRNA-375, and miRNA376a. miRNA34c and miRNA30d were used as controls. There was no difference in the expression profile of the miRNAs between non-encapsulated and encapsulated islets cultured for 1, 3 and 7 days respectively (Fig. 6). The most abundant miRNAs in islets were miRNA-375 and miRNA-7 as has been reported previously [22–24].

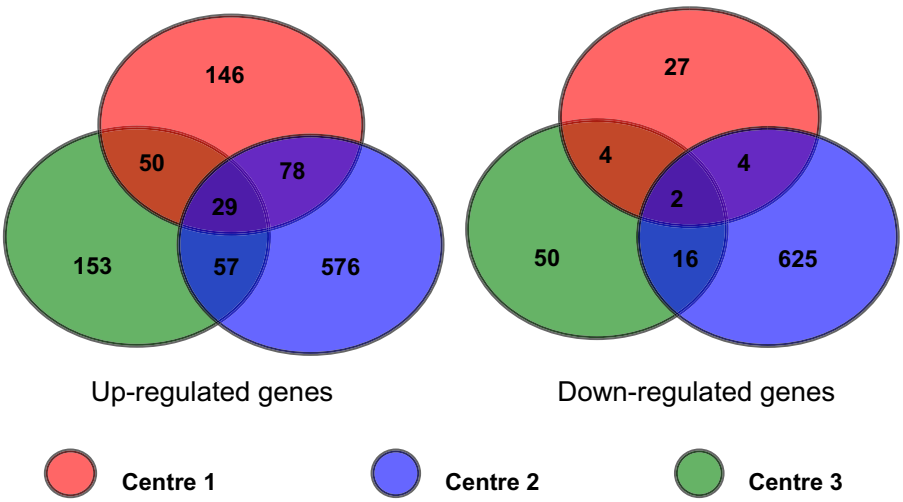

Fig. 3. Venn diagram representing the number of up-regulated and down-regulated genes in encapsulated islets from different centres compared with the corresponding non-encapsulated islets. The results demonstrate that the effect of encapsulation on islets is limited with as few as 29 genes up-regulated and 2 genes down-regulated. Analysis was carried out using the Genespeed/P3 analysis and a cut-off of 0.1.

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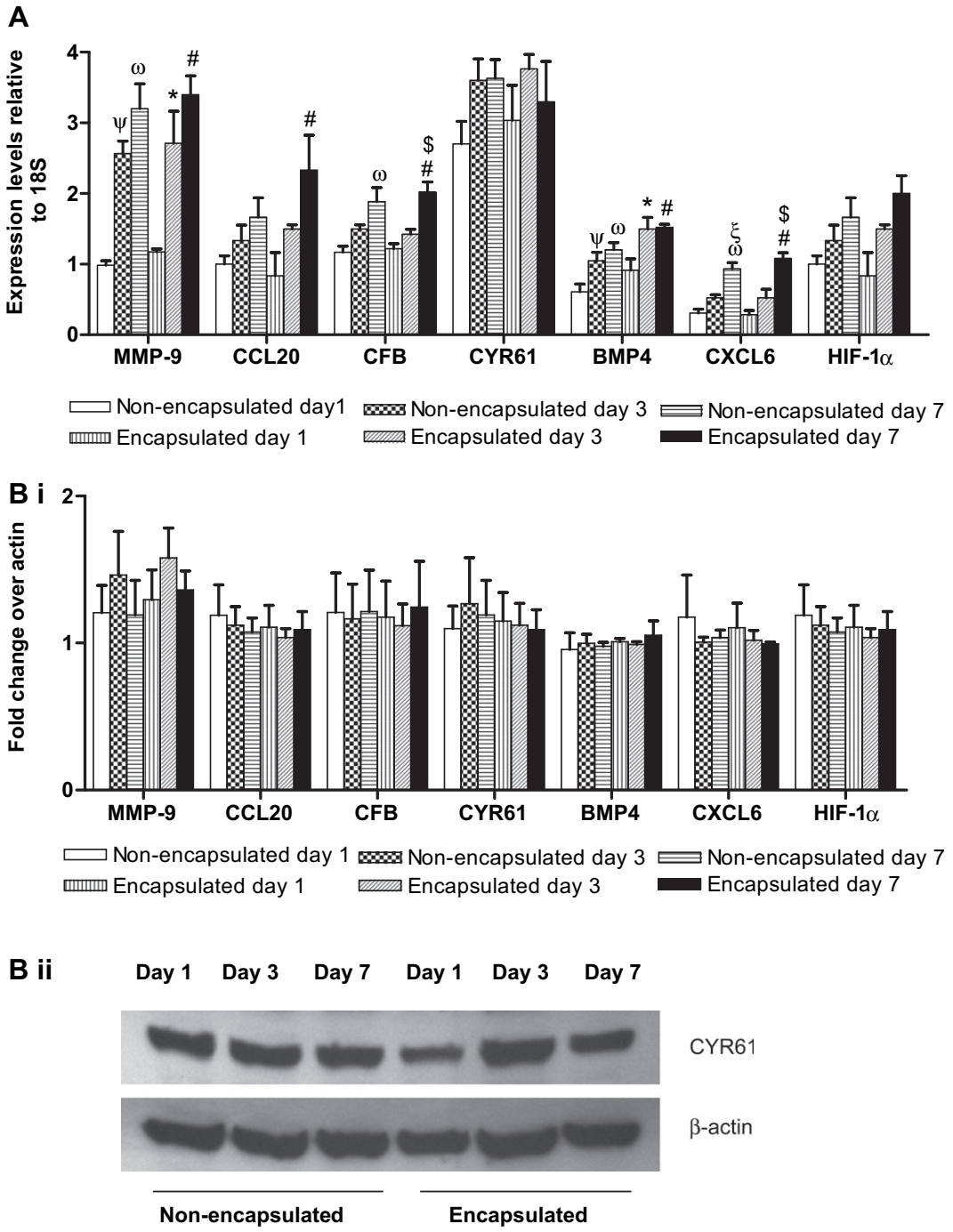

Fig. 4. (A) Expression levels of MMP-9, CCL20, CFB, CYR61, BMP4, CXCL6 and HIF-1α genes of both non-encapsulated and encapsulated islets from different centres by real-time PCR. The mRNA expression of the genes was expressed as relative to the housekeeping gene 18S. There was no significant difference in the gene expression levels comparing non-encapsulated islets with the corresponding encapsulated islets cultured for days 1, 3 and 7 respectively ($P > 0.05$). However, the gene expression levels increased with culture in both non-encapsulated and encapsulated islets. Values = mean ± SEM, ($n = 3$), Non-encapsulated: ω Day 7 > Day 1; ξ Day 7 > Day 3; ψ Day 3 > Day 1, Encapsulated: # Day 7 > Day 1; $ Day 7 > Day 3; * Day 3 > Day 1 (one-way ANOVA, Duncan's multiple comparison test). (B) Protein expression levels of MMP-9, CCL20, CFB, CYR61, BMP4, CXCL6 and HIF-1α both non-encapsulated and encapsulated islets from different centres measured by western blot. (i) Protein levels were expressed as fold changes relative to β-actin. There was no significant difference in the protein levels of both non-encapsulated and encapsulated islets cultured for 1, 3 and 7 days respectively. (ii) Representative western blot picture of the different proteins analyzed. Values are mean ± SEM ($n = 3$).

3.7. Transplantation of encapsulated human islets

To confirm that the encapsulated islets functioned appropriately *in vivo*, they were transplanted into the peritoneal cavity of diabetic immunodeficient mice. Implantation of 3000 IEQs from all 3 centres resulted in normoglycaemia in all mice; however, the time taken to achieve this was shortest in Centre 1, 4.6 ± 0.6 days, and longest in Centre 3, 21.8 ± 1.9 days; Centre 2 was intermediate at 8.0 ± 1.1 days (Fig. 7A). Oral glucose tolerance tests were normal in mice grafted with islets from Centres 1 and 2, but not Centre 3 (Fig. 7B). Implantation of 2000 IEQs from Centres 1 and 2 achieved euglycaemia in all mice grafted;

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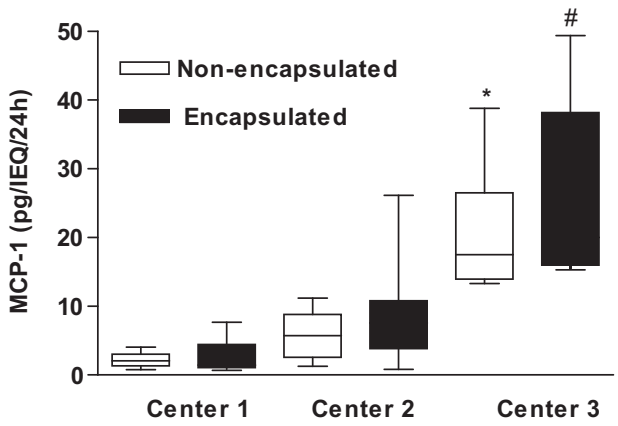

Fig. 5. MCP-1 protein expression of both non-encapsulated and encapsulated islets obtained from different centres. Non-encapsulated and encapsulated human islets were cultured for 1, 3 and 7 days respectively and the MCP-1 levels in culture supernatants measured. MCP-1 levels were the highest in islets from Centre 3 compared to Centres 1 and 2. There was no significant difference in the MCP-1 levels between the corresponding non-encapsulated and encapsulated islets from the three centres cultured for different periods of time. Values = mean ± SEM (data are pooled values from non-encapsulated and encapsulated islets cultured for 1, 3 and 7 days respectively). Centre 1 ($n = 3$); Centre 2 ($n = 5$) and Centre 3 ($n = 3$). *Non-encapsulated islets: Centre 3 > Centres 1 and 2; # Encapsulated islets: Centre 3 > Centres 1 and 2 (ANOVA and Duncan's multiple comparison test).

no mice grafted with this number of IEQs from Centre 3 achieved normoglycaemia (Supplementary Figure 4A and B). 1000 IEQs from Centre 1 normalized blood glucose levels in 40% of diabetic mice; this number of IEQs from Centres 2 and 3 was insufficient to achieve euglycaemia (Supplementary Figure 5A and B). These results indicate differences among the centres in the quality of the islets isolated, a finding noted previously (Fig. 2).

4. Discussion

Several studies have reported the effect of alginate composition and encapsulation on insulin-producing cells placed inside these microcapsules [11,25,26]. However, the scope of these studies has been limited to effects on cell growth, metabolism and insulin secretion. Moreover, there are no reports on the effect of alginate on encapsulated human islets. We believe this is the first study examining the effect of alginate encapsulation on the global gene expression and miRNA expression profile of human islets.

Inability of encapsulated islets to achieve long-term euglycemia in some diabetic recipients may be contributed to by the composition of the alginate [27,28] and the size of microcapsules [29,30]. Whether the process of encapsulation had any effect was unknown prior to our study. We find very few genes that are affected by encapsulation, with almost all of these up-regulated (Supplementary Table 1). These affected were involved mostly in enhancing inflammation, especially by chemotaxis, and vascularisation. Importantly, no genes associated with the survival and function of β-cells were identified to be significantly altered in their expression patterns. Although Q-PCR showed an increase in the expression of genes such as MMP-9, CCL20, CFB, BMP4, CYR61 and CXCL6, this was an effect of culture rather than encapsulation, since similar increases were observed in non-encapsulated islets that were cultured. Moreover, the lack of corresponding increase in functional protein expression raises questions about the biofunctional significance of the gene changes (Fig. 4A and B). Also, no genes associated with hypoxia were identified in the Ingenuity analysis. Since the probe for the hypoxia responsive gene, HIF-1α, was absent from the Illumina bead chip, we carried out Q-PCR for HIF-1α. No difference in expression of HIF-1α was observed between non-encapsulated and encapsulated islets although there was an increase during tissue culture. The functional significance of this is questionable since neither encapsulation nor tissue culture had any effect in expression of HIF-1α protein (Fig. 4A and B); moreover, there was no increase in expression of the genes downstream of HIF-1α, such as VEGF. These data suggest that encapsulated islets, like non-encapsulated islets, may not suffer from hypoxia during *in vitro* culture.

In this study, we used islets isolated from three different centres to overcome the variabilities associated with islet isolation outcomes. The PCA of non-encapsulated islets from different centres and differences in transplantation outcome based on the origin of the islets revealed an effect of the centre of origin on islet quality. Expression of endocrine genes was highest, and exocrine and ductal genes factors lowest, in islets isolated in Centre 1. By contrast, islets from Centre 3 were highest in non-endocrine genes, indicating the purity of the β cells was lowest from this source (Fig. 2). Verification of this finding can be found in the transplant

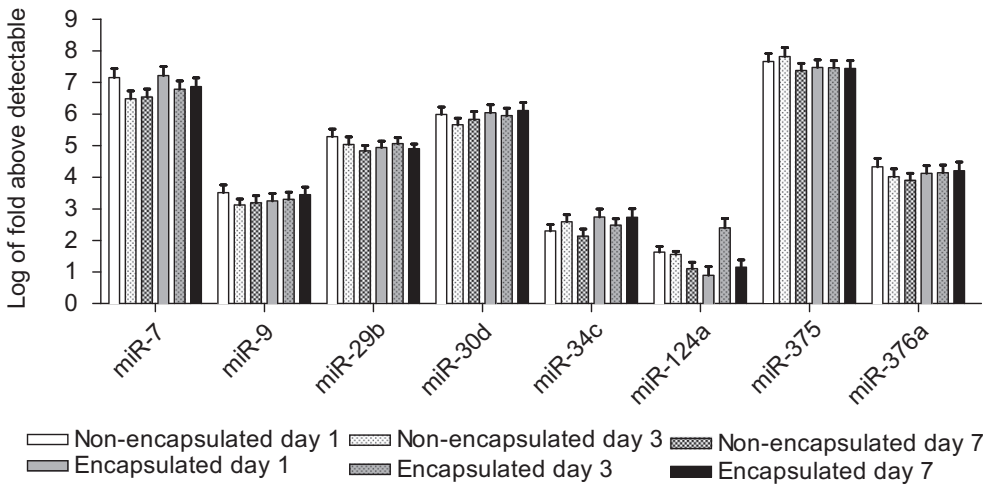

Fig. 6. MicroRNA expression analysis of both non-encapsulated and encapsulated islets by real-time PCR. Six different miRNA expressions which have been described to be essential for β-cell function and development was analyzed. There was no significant difference in the miRNA expression levels between the non-encapsulated and encapsulated islets cultured for days 1, 3 and 7 respectively. Representative picture of non-encapsulated and encapsulated islets obtained from one isolation Centre (Centre 1) ($n = 3$). Identical results were obtained from all the three centres.

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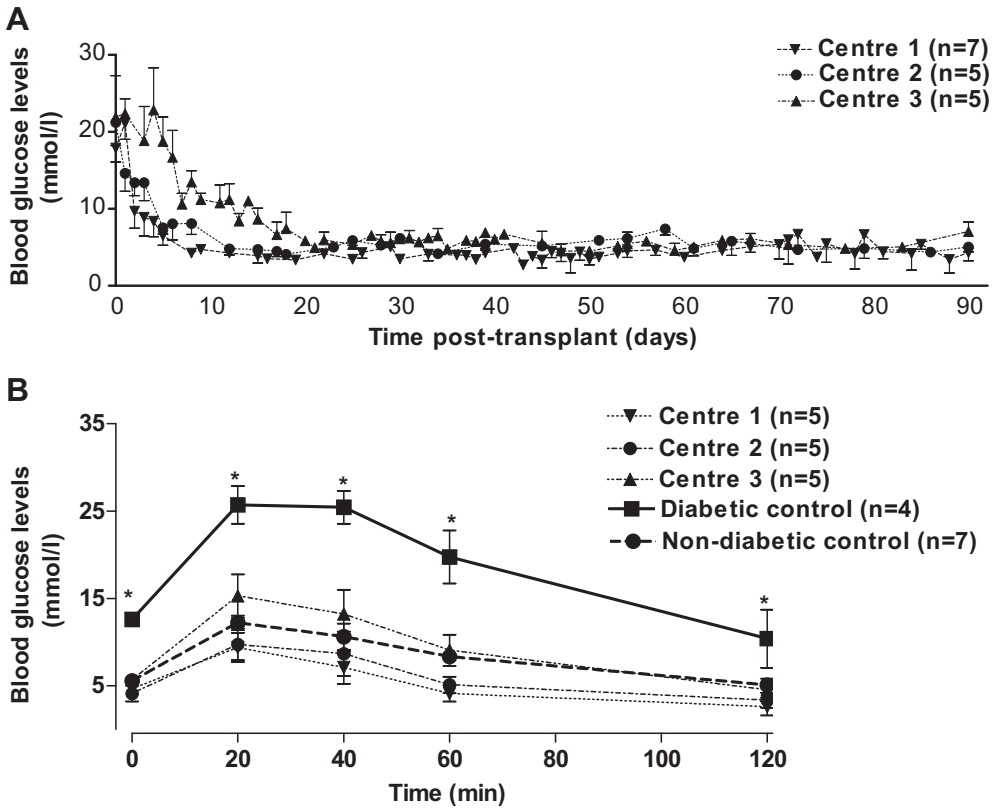

Fig. 7. Transplantation of 3000 IEQs of encapsulated human islets. 3000 IEQs of encapsulated human islets isolated from three different Centres were transplanted into peritoneal cavity of male NOD/SCID mice rendered diabetic by streptozotocin (70 mg/kg). (A) Normoglycemia was achieved in 100% mice however; mice receiving islets from Centre 3 took a long time to achieve euglycemia. (B) Oral glucose tolerance test at 60 days post-transplantation. Values are mean ± SEM. Centres 1, 2, 3 and non-diabetic control < diabetic control *ANOVA and Duncan's multiple comparison test.

outcomes, with more islets from Centre 3 required to achieve lowering of blood glucose levels in recipient diabetic mice than with islets from the other two centres (Supplementary Figures 4 and 5). Key non-endocrine factors in achieving this outcome are the chemokines, especially CCL2/MCP1 and tissue factor, which are produced by exocrine/ductal cells and are toxic to β-cells [18,20,31]. It is believed that short-term culturing of human islets is beneficial as it decreases their immunogenicity, perhaps by decreasing expression of HLA-DR and/or reducing production of toxic chemokines/cytokines [32]. However cultured islets may loose their three-dimensional structure and this is followed by diminished secretion of insulin [33]. Further, the islet recovery rate of encapsulated islets is significantly higher compared to non-encapsulated islets during short and long term *in vitro* culture, with improved graft post-transplantation [34,35]. In this study we have demonstrated that human islets encapsulated in simple barium alginate beads can be cultured for at least a week without affecting islet structure and function (Fig. 1A). Thus encapsulating human islets should enable them to be cultured over long periods, thereby potentially providing a time frame to acquire sufficient numbers of islets from multiple donors for transplantation.

Recently, microRNAs (miRNAs), a class of small endogenous non-coding RNA molecules has been shown to be involved in post-transcriptional regulation of gene expression [36]. A number of miRNAs have been recently shown to be involved in the development and function of pancreatic β-cells and in glucose and lipid metabolism [23]. In this study we found that encapsulation of human islets did not affect the expression profile of miRNAs in human islets, indicating that encapsulation does not alter the expression of these micro-regulators of endocrine pancreas function. Of the miRNAs studied, we found miR-375 and miR-7 had the highest expression in both non-encapsulated and encapsulated islets, exactly as has been reported previously for pancreatic islets and β cells [22,23].

5. Conclusion

We show that alginate microencapsulation does not alter mRNA or miRNA expression of human islets isolated at three different centres in the world. These data suggest that microencapsulation is safe for encasing islets and perhaps other cells [3], at least *in vitro*. Considering that the cellular capsule content (in this case islets) appears biologically unchanged, and remains viable following the procedure, we believe that emphasis should now be towards the capsule/host interface, in order to optimize alginate-based capsule therapies.

Conflict of interest

The lead author VV, received a scholarship from the Australian Foundation for Diabetes Research. MVJ was supported by a fellowship from the Council of Scientific and Industrial Research, Government of India, India.

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Supplementary material

Supplementary material associated with this article can be found in the online version, at doi:10.1016/j.biomaterials.2011.06.044.

References

[1] Shapiro AMJ, Lakey JRT, Ryan EA, Korbutt GS, Toth E, Warnock GL, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 2000;343:230–8.
[2] Vaithilingam V, Sundaram G, Tuch BE. Islet cell transplantation. Curr Opin Organ Trans 2008;13:1–6.
[3] de Vos P, Faas MM, Strand B, Calafiore R. Alginate-based microcapsules for immunoisolation of pancreatic islets. Biomaterials 2006;32:5603–17.
[4] Silva AI, de Matos AN, Brons IG, Mateus M. An overview on the development of a bio-artificial pancreas as a treatment of insulin-dependent diabetes mellitus. Med Res Rev 2006;26:181–2.
[5] Calafiore R, Basta G, Luca G, Lemmi A, Montanucci MP, Calabrese G, et al. Microencapsulated pancreatic islet allografts into nonimmunosuppressed patients with type 1 diabetes: first two cases. Diabetes Care 2006;29:137–8.
[6] Tuch BE, Keogh GW, Williams LJ, Wu W, Foster JL, Vaithilingam V, et al. Safety and viability of microencapsulated human islets transplanted into diabetic humans. Diabetes Care 2009;32:1887–9.
[7] de Groot M, Schuurs TA, van Schilfgaarde R. Causes of limited survival of microencapsulated pancreatic islet grafts. J Surg Res 2004;121:141–50.
[8] de Haan BJ, Faas MM, de Vos P. Factors influencing insulin secretion from encapsulated islets. Cell Transplant 2003;12:617–25.
[9] de Vos P, Van Straaten JF, Nieuwenhuizen AG, de Groot M, Ploeg RJ, De Haan BJ, et al. Why do microencapsulated islet grafts fail in the absence of fibrotic overgrowth? Diabetes 1999;48:1381–8.
[10] Van Schilfgaarde R, de Vos P. Factors influencing the properties and performance of microcapsules for immunoprotection of pancreatic islets. J Mol Med 1999;77:199–205.
[11] Simpson NE, Grant SC, Gustavsson L, Peltonen VM, Blackband SJ, Constantinidis I. Biochemical consequences of alginate encapsulation: a NMR study of insulin-secreting cells. Biomaterials 2006;27:2577–86.
[12] Ricordi C, Lacy PE, Scharp DW. Automated islet isolation from human pancreas. Diabetes 1989;38:1140–2.
[13] Qi M, Strand BL, Mørch Y, Lacík I, Wang Y, Salehi P, et al. Encapsulation of human islets in novel inhomogeneous alginate-Ca^{2+}/Ba^{2+} microbeads: in vitro and in vivo function. Artif Cells Blood Substit Immobil 2008;36:403–20.
[14] Foster JL, Williams G, Williams LJ, Tuch BE. Differentiation of transplanted microencapsulated fetal pancreatic cells. Transplantation 2007;83:1440–8.
[15] Joglekar MV, Patil D, Joglekar VM, Rao GV, Reddy DN, Mitnala S, et al. The miR-30 family microRNAs confer epithelial phenotype to human pancreatic islets. Islets 2009;1:137–47.
[16] Gao SY, Lees JG, Wong JC, Croll TI, George P, Cooper-White JJ, et al. Modeling the adhesion of human embryonic stem cells to poly(lactic-*co*-glycolic acid) surfaces in a 3D environment. J Biomed Mater Res A 2010;92:683–92.
[17] Pfaffl M. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29:2002–7.
[18] Lee I, Wang L, Wells AD, Ye Q, Han R, Dorf ME, et al. Blocking the monocyte chemoattractant protein-1/CCR2 chemokine pathway induces permanent survival of islet allografts through a programmed death-1 ligand-1-dependent mechanism. J Immunol 2003;171:6929–35.
[19] Abdi R, Means TK, Luster AD. Chemokines in islet allograft rejection. Diabetes Metab Res Rev 2003;19:186–90.
[20] Piemonti L, Leone BE, Nano R, Saccani A, Monti P, Maffi P, et al. Human pancreatic islets produce and secrete MCP-1/CCL2: relevance in human islet transplantation. Diabetes 2002;51:55–65.
[21] Schroppel B, Zhang N, Chen P, Chen D, Bromberg JS, Murphy B. Role of donor-derived monocyte chemoattractant protein-1 in murine islet transplantation. J Am Soc Nephrol 2005;16:444–51.
[22] Bravo-Egana V, Rosero S, Molano RD, Pileggi A, Ricordi C, Dominguez-Bendala J, et al. Quantitative differential expression analysis reveals miR-7 as major islet microRNA. Biochem. Biophys. Res. Commun 2008;366:922–6.
[23] Tang X, Tang G, Ozcan S. Role of microRNAs in diabetes. Biochim Biophys Acta 2008;779:697–701.
[24] Joglekar MV, Joglekar VM, Hardikar AA. Expression of islet-specific microRNAs during human pancreatic development. Gene Expr Patterns 2009;9:109–13.
[25] Stabler C, Wilks K, Sambanis A, Constantinidis I. The effects of alginate composition on encapsulated bTC3 cells. Biomaterials 2001;22:1301–10.
[26] Simpson NE, Stabler CL, Sambanis A, Constantinidis I. The role of the CaCl2–guluronic acid interaction on alginate encapsulated bTC3 cells. Biomaterials 2004;25:2603–10.
[27] King A, Andersson A, Strand BL, Lau J, Skjak-Braek G, Sandler S. The role of capsule composition and biologic responses in the function of transplanted microencapsulated islets of Langerhans. Transplantation 2003;76:275–9.
[28] Klock G, Frank H, Houben R, Zekorn T, Horcher A, Siebers U, et al. Production of purified alginates suitable for use in immunoisolated transplantation. Appl Microbiol Biotechnol 1994;40:638–43.
[29] de Vos P, De Haan B, Pater J, Van Schilfgaarde R. Association between capsule diameter, adequacy of encapsulation, and survival of microencapsulated rat islet allografts. Transplantation 1996;62:893–9.
[30] Bunger CM, Gerlach C, Freier T, Schmitz KP, Pilz M, Werner C, et al. Biocompatibility and surface structure of chemically modified immunoisolating alginate-PLL capsules. J Biomed Mater Res 2003;67:1219–27.
[31] Johansson H, Lukinius A, Moberg L, Lundgren T, Berne C, Foss A, et al. Tissue factor produced by the endocrine cells of the islets of Langerhans is associated with a negative outcome of clinical islet transplantation. Diabetes 2005;54:1755–62.
[32] Gaber AO, Fraga D. Advances in long-term islet culture: the memphis experience. Cell Biochem Biophys 2004;40:49–54.
[33] Schmied BM, Ulrich A, Matsuzaki H, Ding X, Ricordi C, Weide L, et al. Transdifferentiation of human islet cells in a long-term culture. Pancreas 2001;23:157–71.
[34] Korbutt GS, Mallett AG, Ao Z, Flashner M, Rajotte RV. Improved survival of microencapsulated islets during in vitro culture and enhanced metabolic function following transplantation. Diabetologia 2004;47:1810–8.
[35] Zhao M, Christie MR, Heaton N, George S, Amiel S, Cai HG. Amelioration of streptozotocin-induced diabetes in mice using human islet cells derived from longterm culture in vitro. Transplantation 2002;73:1454–60.
[36] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281–97.