

Electronic Supplementary Material

Methods and Results

Human islet endothelial cells preparation and characterization

Human islets were obtained from the pancreas of organ donors using a modification of Ricordi's technique as described [1]. Approximately 100,000 human islets, 85-90% pure as assessed by dithizone staining, were incubated at 37°C in RPMI (Life Technologies, Paisley, UK) supplemented with collagenase type I-A (1mg/ml; Sigma Aldrich, UK). To remove larger debris, the digest was passed through a 70 µm filter, and the remaining cell suspension was washed twice at 300g for 10 minutes in RPMI/2.5% foetal calf serum (FCS, Euroclone, Paignton, Devon, UK). The resultant cell pellet was resuspended in 0.25% trypsin/1 mmol EDTA (Life Technologies) for 10 minutes at 37°C to obtain a single cell suspension, washed and resuspended in 80 µl of PBS/0.5% FCS, with 20 µl of anti-CD105 immunomagnetic beads (DynaI, Wirral, Merseyside, UK). After 15 minutes at 6°C, cells were washed, resuspended in appropriate buffer and positively selected using a MP3 magnetic cell particle separator (DynaI), according to the manufacturer's instructions. Finally, the enriched islet MECs were resuspended in MCDB131 medium (Life Technologies) with 20% FCS, 10 mmol L-glutamine, 12 µg/ml ECGF, 10 ng/ml EGF, 1µg/ml hydrocortisone (all products from Clonetics Corp., San Diego, CA) and antibiotics (100 IU/ml penicillin, 100 µg/ml streptomycin) (Sigma, Milan, Italy), onto ECAF (Sigma) coated tissue culture plates. Cells were grown to confluence and analysed for the expression of endothelial cell surface markers. If necessary, the positive immunomagnetic bead selection was repeated to achieve more than 90% purity of the islet MEC culture.

Cells were grown until confluent, with medium changing every 48-72 hours, washed twice and dispersed with trypsin/EDTA as required for analysis or subculture (splitting cells 1:2 or 1:3) (ESM Figure 1a).

Endothelial phenotype was assessed by flow cytometric analysis of the cell surface

phenotype, staining for basal expression of endothelial markers, or of permeabilized cells for intracellular vWF staining, as described [2]. Cells were stained at RT with saturating amounts of anti-human mAb against CD105-RPE (undiluted, Serotec Ltd, Oxford, UK), CD31-RPE (undiluted, Serotec), VE-cadherin (dilution 1:30) (BD PharMingen, San Diego, CA), CD146-RPE (clone P1H12, dilution 1:100) (Chemicon Int., Temecula, CA), E-selectin-FITC (CD62E, dilution 1:20), (all from Serotec), and rabbit anti-human vWF antiserum (dilution 1:200) (Sigma). After washing, RPE-conjugated F(ab')₂ fragment of goat anti-mouse immunoglobulins or FITC-conjugated anti-rabbit immunoglobulins (Dako, Glostrup, DK) were used as secondary antibodies. Ten thousand events were collected and results were expressed as MFI and percentage of positive gated events, with gates established using appropriate isotype control antibodies. In all experiments, cells were also stained with the corresponding isotype control antibodies and non-specific staining subtracted from the appropriate population. Expression of vWF was also evaluated by immunofluorescence microscopy (ESM Figure 1a) [2].

In vitro angiogenesis assay was performed to determine whether islet MECs were capable to align to form cord-like structures, also defined as capillary-like, they were cultured on polymerised Matrigel, a basement membrane-like matrix. Matrigel was applied to glass slides and allowed to gel at 37° C. Primary islet MECs were seeded onto the Matrigel, cultured and observed for 1-24 hour for formation of cord-like structures by microscopy. Cord formation was imaged by phase-contrast microscopy, and representative fields were photographed (Windows MicroImage, ver. 3.4 CASTI Imaging, I) using a low-light video camera (ESM Figure 1c).

The immortalization of the islet endothelial cells was carried out by using a chimeric adeno5/SV40 virus [3]. The recombinant virus was achieved by cloning SV40 large T-Ag DNA in adenovirus type 5 vector. A 25 cm² flask of islet MECs at the third passage after isolation and purification was infected with the purified chimeric virus for 2 hours at 37°C in serum-free medium at the multiplicity of infection of 200. The infected islet MECs were washed, incubated overnight in complete medium, and finally subcultured. At 28 days post-infection SV40-induced foci were identified, individually detached and propagated. The cells derived from these foci were characterised in order to control the expression of endothelial markers (ESM Figure 1b and 1d). The SV40-induced cell line was established by subculturing the cells every 5 days at a split ratio 1:2 [4]. These immortalised cells were shown to represent a substitute for the primary counterparts, retaining their characteristics. Expression of vWF and endothelial-cell associated markers were repeatedly reassessed after every 3 to 4 subcultures.

GHSR1A, GLP1R, GPR39 receptor expression

GHSR1A, GLP1R, GPR39 mRNA expression were assessed by standard polymerase chain reaction (PCR) as described in a 50 µL volume using AmpliTaq Gold Polymerase in a GeneAmp PCR System (Perkin Elmer, Italy) under the following conditions: 94°C for 5 minutes, followed by 35 cycles consisting of 94°C for 30 seconds, annealing for 30 seconds, and 72°C for 30 seconds, followed by 7 minutes at 72°C for final elongation [5, 6]. The annealing temperature was adjusted for each target: 59°C for *GHSR1A*, 54°C for *GPR39*, 56°C for *GLP-1R*. The primer sequences were: human *GHSR1A*: forward 5'-TCAGGGACCAGAACCAC-3'; reverse 5'-GCCAGGCTCAAAGGAT-3' (accession number U60179); human *GPR39*: forward 5'- 3'; reverse 5'-3' (accession number: AF034633); human *GLP1R*: forward 5'-TTCTGCAACCGGACCTTCGA-3' and reverse 5'-ATGAGTGTGTCAGCGTGGACGTTG-3'; (accession number: NM_002062.3).

Quantitative real-time PCR was performed using the following primer sequences: human

GLP1R forward 5'TCAGAGACGGTGCAGAAATG, reverse 5'CAGCTGACATTACGAAGGA;
human *GHSR1A* forward TCTCATCGGCAGGAAGCTG, reverse
CCAGCAGAGGATGAAGGCA, *GPR39* forward GACCATCATCTTCCTGAGGCTG and
reverse GAGAAGGGGAGGAGGATCATGTA.

To eliminate traces of genomic DNA, RNA samples were treated with DNA-free DNase according to manufacturer instructions (Ambion). Real-time PCR was conducted with the SYBR Green chemistry in 25 µl volume containing 50 ng of cDNA, 150 nM primers and a 2x master mix (Fermentas, BioRad), according to the conditions recommended by the supplier. Housekeeping gene 18S rRNA was used as internal standard. Results were reported as gene expression relative to a chosen positive control (brain), and relative quantification analysis was performed with the 7300 Sequence Detection System Software (Applied Biosystems, Monza, I), using the comparative cycle threshold (Ct) ($2^{-\Delta\Delta C_t}$) method.

Surface expression of GHS-R1a and GPL-1R was assessed by IF. Chamber slide attached cells at subconfluent density were fixed with 3% paraformaldehyde, subsequently blocked and permeabilized in PBS, 5% BSA, 0.1% Triton for 1 hour. Cells were then incubated with anti-GHS-R1a Ab (1/100) or anti-GLP-1R Ab (1/100) (Santa Cruz Biothecnology, Heidelberg, Germany) in PBS, 2%BSA, 0.01% Triton ON at 4°C. Cell were then washed and incubated with Alexa Fluor 488 anti-goat or anti-rabbit IgG (Molecular Probes, Leiden, NL) as secondary Ab for 1 h at RT. Hoechst 33258 (Sigma) dye was used for nuclear staining, and slides were mounted in Vectashield H-1000 mounting medium (Vector Laboratories, Burlingame, CA). Confocal analysis was performed using a Zeiss LSM5 Pascal Model confocal microscope (Carl Zeiss International, Germany) using a 630X magnification lens. Control experiments included incubation with nonimmune isotypic control Ab or the omission of primary Ab.

IF studies were also performed on cryostatic sections of pancreas tissue obtained from organ donors. Three different donors were used. After 5 min. fixation in 3.5% paraformaldehyde, sections were incubated with anti-GHS-R1a Ab (1/100) or anti-GLP-1R Ab (1/100), and double stained with

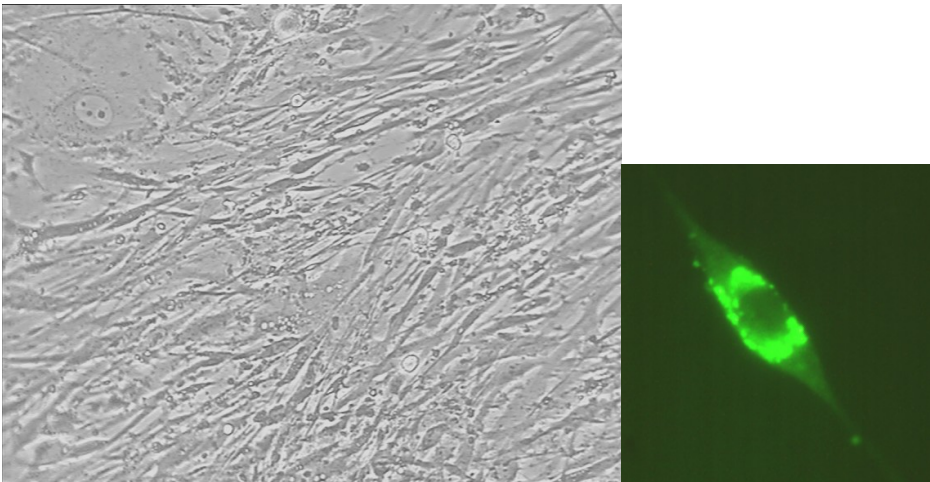
anti-CD31 (1/50) for 1 hour at RT. As secondary antibodies Alexa Fluor 488 anti-goat or anti-rabbit IgG, and Alexa Fluor 594 anti-mouse IgG were used where appropriated. Confocal analysis was performed as above using a 400X magnification.

References

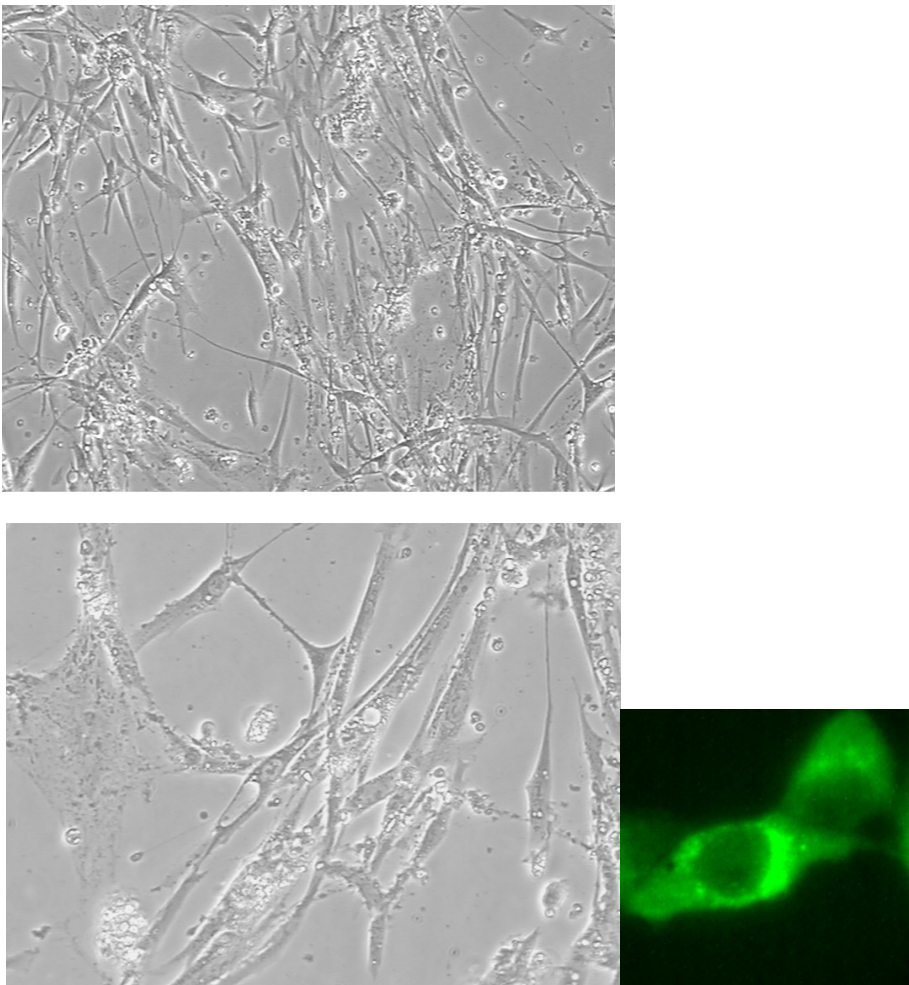
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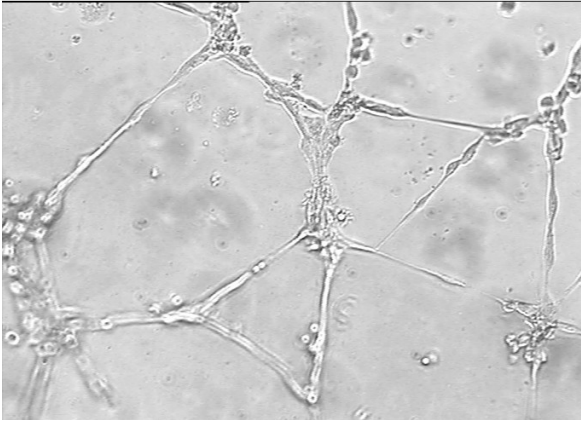
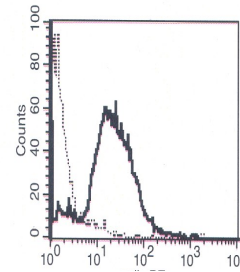
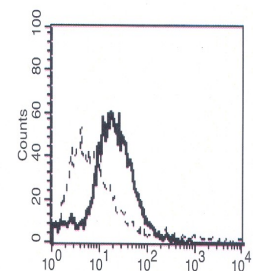
ESM Figure 1

a



b



c**d****CD31****CD105**

Representative morphological analysis of islet MECs.

Light microscopy of cultured primary **(a)** and immortalised **(b)** islet MECs observed by phase contrast (original magnification 100X and 200X). The cells exhibit an elongated shape, sometimes with more granular appearance for the immortalised ones. Islet MECs retain endothelial cell characteristics, i.e. positive cytoplasm staining for vWF, assessed by immunofluorescence.

(c) Islet MECs cultured on Matrigel for 24 hours aligned to form capillary-like structures (representative phase contrast micrograph).

(d) Representative cytofluorimetric detection of staining for CD31 and endoglin (CD105). Thin lines represent the corresponding isotype control antibodies.