

E. Favaro · A. Bottelli · B. Lozoska-Ochser ·  
E. Ferioli · G. C. Huang · N. Klein · A. Chiaravalli ·  
P. Cavallo Perin · G. Camussi · M. Peakman ·  
P. G. Conaldi · M. M. Zanone

## Primary and immortalised human pancreatic islet endothelial cells: phenotypic and immunological characterisation

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**Abstract** *Aims/hypothesis:* Studies on the biology of the microvascular endothelial cells (MECs) that surround and penetrate the pancreatic islets are hampered by difficulties in isolating and culturing large numbers of pure cells. We aimed to morphologically and functionally characterise primary MECs purified and cultured from human islets, and to establish a simian virus 40 (SV40)-immortalised cell line from these primary cultures. *Materials and methods:* Human islet MECs were extracted and purified using anti-CD105 coated immunomagnetic beads, and endothelial markers and surface molecules analysed by flow cytometric analysis. An immortalised cell line was then established by using a chimeric adeno5/SV40 virus. *Results:* Islet MECs expressed classic and specific endothelial markers, a high basal level of intercellular adhesion molecule-1, and low levels of E-selectin and TNF (previously known as TNF- $\alpha$ ) inducible vascular cell

adhesion molecule-1. IFNG (previously known as IFN- $\gamma$ ) induced expression of HLA class II molecules. The immortalised islet MECs expanded rapidly, exhibited increased DNA synthesis, and were passaged approximately 30 times, without signs of senescence. They retained the endothelial characteristics of the parental cells, and behaved as the primary cells in terms of TNF stimulation of expression of adhesion molecules and support of leucocyte adhesion and transmigration. *Conclusions/interpretation:* The immortalised islet MECs that we have established could effectively represent a substitute for primary counterparts for in vitro studies on the role of the microvasculature in pathophysiological processes involved in type 1 and type 2 diabetes.

**Keywords** Adhesion molecules · Endothelial cells · Islets of Langerhans · Large T antigen · SV40

**Abbreviations** Api:  $\alpha$ -1 proteinase inhibitor · BrdU: 5-bromo-2'-deoxyuridine · ICAM1: intercellular adhesion molecule-1 · MECs: microvascular endothelial cells · MFI: mean fluorescence intensity · PBMCs: peripheral blood mononuclear cells · RPE: R phycoerythrin · SV40: simian virus 40 · T-Ag: T antigen · VCAM1: vascular cell adhesion molecule-1 · vWF: von Willebrand's factor

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E. Favaro · P. C. Perin · G. Camussi · M. M. Zanone (✉)  
Department of Internal Medicine and Center of Experimental  
Medicine (CeRMS), University of Turin, Turin, Italy  
e-mail: mmz@libero.it  
Tel.: +39-011-6336708  
Fax: +39-011-6631184

A. Bottelli · E. Ferioli · A. Chiaravalli · P. G. Conaldi  
Departments of Medicine and Public Health and Pathology,  
University of Insubria, Varese, Italy

B. Lozoska-Ochser · G. C. Huang · M. Peakman  
Departments of Immunobiology and Diabetes and Internal  
Medicine, Guy's, King's and St. Thomas's School of Medicine,  
London, UK

N. Klein  
Department of Immunobiology, Institute of Child Health,  
London, UK

M. M. Zanone  
Dipartimento di Medicina Interna,  
Corso Dogliotti 14,  
10126 Torino, Italy

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### Introduction

In addition to mediating transport of nutrients and metabolites, vascular endothelium plays a pivotal role in angiogenesis, haemostasis, and vascular permeability [1]. Through expression and secretion of an array of key immunological accessory molecules and mediators, the microvasculature also behaves as an active 'gatekeeper', in the control of leucocyte recruitment into the tissues and in the mediation of inflammation [2]. A deeper knowledge of tissue-derived endothelium might help us understand its physiological properties and the pathological processes occurring within a specific organ. It is, in fact, widely

accepted that phenotype and function of microvascular endothelial cells (MECs) derived from different vascular beds are heterogeneous [3–8], supporting the proposal that tissue-specific vascular beds have specialised functions.

Against this background, pancreatic islet MECs exhibit distinctive structural features, reminiscent of the appearance of the renal glomerulus. The islet microcirculation is characterised by a dense network of sinusoidal capillaries with a distinctive fenestrated endothelium [9–13], branching from arterioles entering the islet. Each endocrine cell is in close proximity to an endothelial cell; this facilitates a rapid exchange of signals and substances. MECs are important in the delivery of oxygen and nutrients to endocrine cells, and also induce insulin gene expression during islet development [14], as well as providing secretory signals from other cells [15]. Importantly, the fenestrated islet microvascular endothelium not only facilitates rapid release of insulin into the circulation, but also appears to play a role in fine-tuning blood glucose sensing and regulation [12, 16–18]. Moreover, some studies support the possibility that the islet microvasculature participates in sensing the environment of the islets and generating signals to induce the growth and maintenance of the islets in response to physiological stimuli, such as insulin resistance [17–19]. Specific markers of the islet microvasculature are  $\alpha$ -1 proteinase inhibitor (Api) [11] and nephrin, a highly specific barrier protein [19], both of which are absent in other microvascular endothelial districts.

Intra-islet MECs are therefore likely to play a pivotal role in islet physiology and the pathogenesis of types 1 and 2 diabetes. Human and murine studies indicate that during autoimmune insulinitis in type 1 diabetes the islet MECs adopt an activated phenotype and are probably involved in regulating mononuclear cell accumulation in the islets; these cells hyperexpress adhesion molecules, such as intercellular adhesion molecule 1 (ICAM1), PNA<sub>d</sub> and MAdCAM-1 (L-selectin ligands), as well as class II HLA molecules that are important in allowing mononuclear cells to transmigrate and home to pancreatic islets [20–24]. In nonobese diabetic mice, presentation of antigen by endothelial cells has been shown to be required for the homing of insulin-specific diabetogenic CD8<sup>+</sup> T cells [25].

Most of our knowledge on the active participation of endothelial cells in physiopathological processes derives from studies on endothelial cells derived from umbilical vein, which are easily isolated and cultured [26]. However, it is conceivable that these large-vessel endothelial cells do not exhibit the phenotypic and functional characteristics of the relevant microvasculature [3–8, 27]. Studies on the biology of microvascular endothelial cells are hampered by difficulties in isolating and culturing large numbers of pure cells from any organ, and by their limited life span. Easier availability of endothelial cells would therefore facilitate definition of their role at specific sites, and enhance our understanding of the role of the microendothelium in pathophysiological processes, allowing questions to be addressed *in vitro* that are difficult or impossible to address *in vivo*.

The above observations prompted the present study, in which we describe the morphological and functional characterisation of primary MECs purified and cultured from human islets, and the establishment of a simian virus 40 (SV40)-immortalised cell line from these primary cultures.

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## Subjects, materials and methods

### Islet endothelial cell isolation, purification and culture

Human islets were obtained from the pancreas of an organ donor using a modification of Ricordi's technique [28]. Islet isolations were carried out in the Cell Isolation Unit at King's College Hospital, London. These studies were carried out after obtaining informed consent and were approved by the local Ethical Review Committee.

Approximately 100,000 freshly obtained human islets that were 85 to 90% pure as assessed by dithizone staining were incubated at 37°C in RPMI (Invitrogen, Paisley, UK) supplemented with collagenase type I-A (1 mg/ml; Sigma Aldrich, Poole, Dorset, UK) and shaken every 15 min for 2 h. To remove larger debris, the digest was passed through a 70- $\mu$ m filter, and the remaining cell suspension was resuspended and washed at 300 $\times$ g for 10 min in RPMI/2.5% FCS (Invitrogen). To obtain a single cell suspension, the resultant cell pellet was resuspended in 0.25% trypsin/1 mmol/l EDTA (Invitrogen) for 10 min at 37°C, washed and resuspended in 80  $\mu$ l of PBS/0.5% FCS, supplemented with 20  $\mu$ l of anti-CD105 immunomagnetic beads (Dyna, Wirral, Merseyside, UK). After 15 min at 6°C with an end-over-end rotation, cells were washed, resuspended in appropriate buffer and positively selected using an MP3 magnetic cell particle separator (Dyna) according to the manufacturer's instructions. Finally, the enriched islet endothelial cells were resuspended in MEC growth medium with 20% FCS, 10 mmol/l L-glutamine, 12  $\mu$ g/ml endothelial cell growth factor, 10 ng/ml epidermal growth factor, 1  $\mu$ g/ml hydrocortisone (all from TCS CellWorks, Buckinghamshire, UK) and antibiotics (100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin), and transferred onto tissue culture plates coated with endothelial cell attachment factor (Sigma). Cells were grown to confluence, subcultured and analysed for the expression of endothelial cell markers [19].

### Establishment of the immortalised islet endothelial cell line

The immortalisation of the islet endothelial cells was effected using a chimeric adeno5/SV40 virus [29]. The recombinant virus was achieved by cloning SV40 large T antigen (T-Ag) DNA in adenovirus type 5 vector. A 25-cm<sup>2</sup> flask of islet MECs at the third passage after isolation and purification was infected with the purified chimeric virus for 2 h at 37°C in serum-free medium at a 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. The SV40-induced cell line was established by subculturing the cells every 5 days at a split ratio of 1:2.

The transformation of the immortalised cells was evaluated by assessing their ability to form colonies in soft agar. Moreover, to assess their tumorigenic potential *in vivo*,  $1 \times 10^6$  cells were resuspended in 200  $\mu$ l of medium, chilled on ice, added to 200  $\mu$ l of Matrigel (Sigma) at 4°C and injected subcutaneously into the left back of SCID mice (Charles River, Jackson Laboratories, Bar Harbor, ME, USA) via a 26-gauge needle using a 1-ml syringe.

The possibility that these cells might produce adenovirus-SV40 recombinant virions was also considered by detecting SV40 large T-Ag sequences (see below) in DNA samples extracted from culture supernatants that had been precipitated with polyethylene glycol 8,000 (10%) in NaCl 0.4 mol/l, in order to concentrate any recombinant virions released by the immortalised cells.

#### Detection of SV40 large T-Ag in immortalised islet MECs

The expression of SV40 large T-Ag in the nuclei of the immortalised islet MECs was revealed by immunocytochemical staining. The cells were cultured in chamber slides for 24 h at 37°C and then fixed in 10% paraformaldehyde for 15 min at room temperature. The cell monolayers were incubated with 3% hydrogen peroxide for 10 min at room temperature, treated twice in a microwave oven at 700 W for 5 min, and washed in TBS pH 7.4 and 2% Triton X-100; then, the cells were incubated overnight at 4°C with 4  $\mu$ g/ml of monoclonal antibody specific to SV40 large T-Ag (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing with TBS-Triton the cultures were treated for 1 h at room temperature firstly with biotinylated anti-mouse Igs and then with streptavidin conjugated to horseradish peroxidase. Finally, the cells were stained with 0.03% benzidine and counterstained with haematoxylin and eosin.

To reveal the presence of the complete transcript of SV40 large T-Ag, 1  $\mu$ g of total RNA was reverse transcribed using a first strand synthesis kit (Boehringer Mannheim, Indianapolis, IN, USA). The PCR mixture contained 1  $\mu$ g of cDNA, buffer with nucleotides (200  $\mu$ mol/l each), 1.5 mmol/l MgCl<sub>2</sub>, 1 U of Taq polymerase (Invitrogen), and 0.2  $\mu$ mol/l of specific primers (forward primer: 5'-ATGGA TAAAGTTTTAAAC-3'; reverse primer: 5'-TGAGGTT CAGGGGGAGGT-3'). The reaction was carried out for 40 cycles (30 s at 94°C, 40 s at 53°C and 30 s at 72°C), and the amplicon of 2,124 bp was analysed in a 1.5% agarose gel. cDNA from primary cells and the vector with cloned large T-Ag served as controls.

SV40 large T-Ag DNA sequences were also detected by semi-nested PCR, as described [30]. In the first round of PCR, 1  $\mu$ g of DNA was amplified using 0.2  $\mu$ mol/l of specific primers (Invitrogen, Carlsbad, CA, USA) (forward primer: 5'-CTTTGGAGGCTTCTGGGATGCAACT-3'; reverse primer: 5'-GCATGACTCAAAAACTTAGCAATTCTG-3'). Using the same forward primer and a different reverse primer (5'-GAAAGTCTT TAGGGTCTTCTACC-3'; Invitrogen), 10  $\mu$ l of first-round PCR products were re-amplified. The amplicons (first-round: 575 bp; second-round: 543 bp) were analysed in a 2% agarose gel. DNA from primary cells and SV40-immortalised human podocytes served as controls.

#### Phenotypic characterisation, detection of surface molecules, and immunofluorescence studies

The endothelial phenotype of primary and immortalised islet MECs was assessed by flow cytometric analysis, staining for basal expression of endothelial markers and adhesion molecules, with cell aliquots permeabilised for intracellular staining, as described [19].

Cells were stained at room temperature with saturating amounts of anti-human monoclonal antibody against CD105 conjugated to R phycoerythrin (RPE) (undiluted; Serotec, Oxford, UK), CD31-RPE (undiluted; Serotec), VE-cadherin (dilution 1:30; BD PharMingen, San Diego, CA, USA), CD146-RPE (clone P1H12, dilution 1:100; Chemicon, Temecula, CA, USA), E-selectin-FITC (CD62E, dilution 1:20), ICAM1 (CD54, 1:10), vascular cell adhesion molecule-1 (VCAM1) (CD106, 1:20) (all from Serotec), CD40-FITC (undiluted; Euroclone, Devon, UK), CD154-FITC (undiluted; Euroclone), and rabbit anti-human von Willebrand's factor (vWF) antiserum (dilution 1:200; Sigma).

After washing, RPE-conjugated F(ab')<sub>2</sub> fragment of goat anti-mouse immunoglobulins or FITC-conjugated anti-rabbit immunoglobulins (Dako, Glostrup, DK) were used as secondary antibodies. Cells were analysed by flow cytometry using CellQuest software (BD Biosciences, Erembodegem, Belgium), and results expressed as mean fluorescence intensity (MFI) and/or percentage of positive gated events, with gates established using appropriate isotype control antibodies (Serotec or PharMingen). For the adhesion molecules, expression is presented as the mean of five separate flow cytometric analyses. Expression of E-selectin, ICAM1, VCAM1, CD40 and CD154 was also re-analysed after incubation with recombinant human TNF (previously known as TNF- $\alpha$ ) 10 ng/ml (Sigma) for 4 to 12 h.

For HLA class II molecule staining, cells were incubated for 30 min at 4°C with mouse anti-human antibodies against HLA-DR (BD PharMingen) and analysed at baseline and at 24, 48 and 72 h after stimulation with 100 IU/ml IFNG (previously known as IFN- $\gamma$ ).

Immunofluorescence staining for nephrin on cultured islet MECs was performed as previously described [19].

For intracellular staining of Api<sub>1</sub> cells, permeabilised with 0.5% Triton X-100, were stained with goat anti-human Api polyclonal antibody (1:50) (Rockland, Gilbertsville, PA, USA) as described [11].

#### Proliferation assay

Cells were seeded into 96-well plates and cultured for 48 h. DNA synthesis was then measured by a cell proliferation 5-bromo-2'-deoxyuridine (BrdU)ELISA (Roche Diagnostics, Mannheim, Germany), following the manufacturer's instructions. This is a colorimetric immunoassay, based on the measurement of BrdU incorporation during DNA synthesis. Eight separate experiments were performed, each in triplicate. Data were expressed as percentage change amongst immortalised vs primary MECs (mean Abs<sub>450nm</sub> immortalised MECs/mean Abs<sub>450nm</sub> primary MECs x 100).

#### In vitro angiogenesis assay

To determine whether islet MECs were able 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 and immortalised islet MECs were seeded on to the Matrigel, cultured and observed by microscopy for 1 to 24 h to check for formation of cord-like structures. Cord formation was imaged by phase-contrast microscopy, and representative fields were

photographed (Windows MicroImage, 3.4; CASTI Imaging, Venice, Italy) using a low-light video camera.

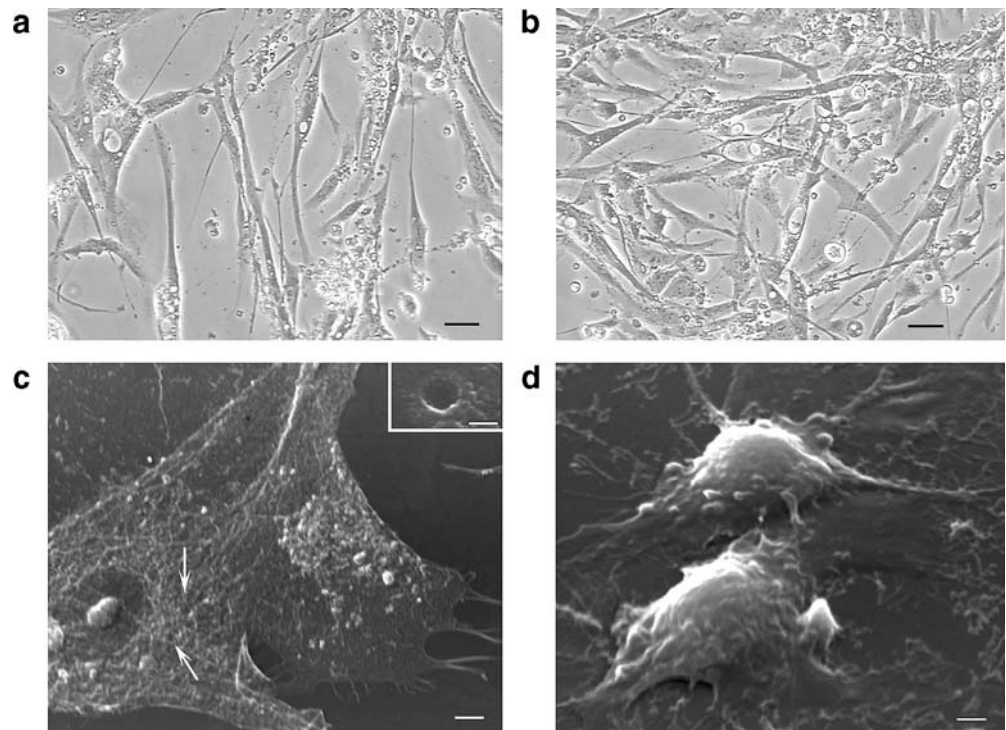
#### Scanning electron microscopy

Cells (approximately 10<sup>4</sup> cells in 200 µl of medium) were seeded on gelatin-coated eight-well chamber slides (Nal-gene Nunc International, Rochester, NY, USA) and cultured for 24 to 48 h to subconfluence. Cells untreated or treated with TNF (10 ng/ml) for 1 h were fixed in Karnovsky's fixative for 1 h, dehydrated in alcohol, dried and coated with gold by sputter coating (Emitech, Ashford, UK). They were then examined in a scanning electron microscope (JSM T300; Jeol, Tokyo, Japan) operating at 25 kV.

#### Mononuclear cell adhesion and transwell migration assays

Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque centrifugation of heparinised blood from a healthy donor, washed in Hanks' balanced salt solution and the pellet resuspended in endothelial cell medium at a concentration of 1×10<sup>6</sup> cells/ml. To measure PBMC adhesion to islet MEC monolayers, 1×10<sup>6</sup> PBMCs in 1 ml of medium were added to each well of confluent cells and incubated for 1 h at 37°C. Non-adherent cells were removed by aspiration of the supernatant and two washes. Endothelial cells and adherent PBMCs were dispersed by incubation with non-enzymatic cell dissociation solution

**Fig. 1** Representative morphological analysis of primary and immortalised islet MECs. Light microscopy of cultured primary (a) and immortalised (b) islet MECs observed by phase contrast (original magnification ×200). The cells exhibited an elongated shape. Immortalised cells sometimes had a more granular appearance, and retained a similar morphology. Bars: 10 µm. c, d Representative micrographs created by scanning electron microscopy of untreated primary islet MECs (c) or of MECs treated with TNF (10 ng/ml) for 1 h (d). TNF treatment induced marked retraction of the cells, ruffling of the surface, and loss of reciprocal contacts; nuclei and nucleoli were no longer visible. Arrows indicate cellular fenestrations (original magnification ×1,500). Bars: 1 µm. Inset (c): representative magnification of a cellular fenestra (original magnification ×15,000). Bar: 0.1 µm



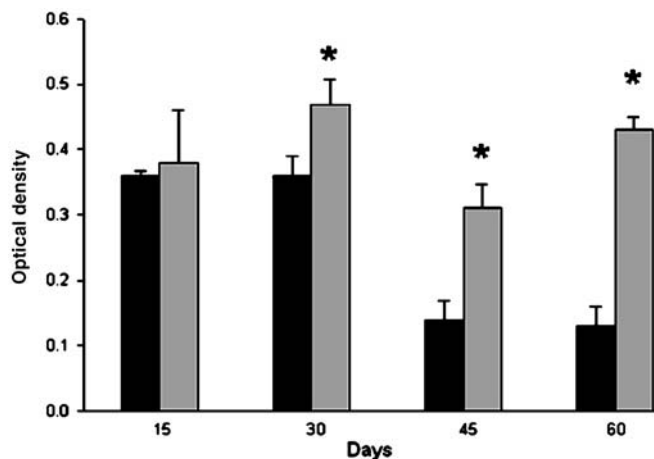
and then washed. The cell pellet was resuspended and aliquoted for staining for all PBMCs and monocytes (with 10  $\mu$ l of a mix of anti-human CD45-FITC and anti-CD14-RPE monoclonal antibodies; Dako) and isotype control for 30 min at 4°C. It was then washed and analysed by immunofluorescent staining and flow cytometry.

In parallel experiments, PBMCs were labelled for 5 min with 4  $\mu$ mol/l PKH2 (Sigma), a green fluorescent membrane stain, following the manufacturer's instructions, and subsequently  $1 \times 10^6$  PBMCs were added to cell monolayers as above. Adherent cells were counted by digital analysis (Windows MicroImage) of images, using a video camera, and expressed as the mean of cells counted in ten  $\times 200$  inverted microscope fields.

To evaluate whether PBMC adhesion could be upregulated by TNF, primary and immortalised cell monolayers were treated with TNF (10 ng/ml) for 12 h, and adhesion experiments performed as above. All the adhesion studies were carried out using duplicate wells, and three different sets of experiments were performed.

In migration experiments, 3- $\mu$ m pore size polyethylene transwells (Corning Costar, MA, USA) were prepared by coating transwell inserts with endothelial cell attachment factor. Endothelia were seeded at  $2 \times 10^5$  cells per transwell insert and allowed to grow to confluence. Endothelial integrity was assessed by a trypan blue-albumin permeability assay, sampling aliquots of the bottom well and measuring absorbance at 590 nm, as described [31]. PBMCs, at a concentration of  $5 \times 10^5$  per well, were added to the upper chamber of the transwell. PBMCs migrating into the lower chamber were quantitated by direct sampling and counting in a haemocytometer at 1, 4 and 18 h. In parallel experiments, transwells were incubated in the presence of TNF (10 ng/ml) for 4 and 12 h.

All the transwell studies were carried out using duplicate wells and counting of the cells in the lower chamber was in triplicate. Results were expressed as a percentage of the starting cell numbers.



**Fig. 2** Time course proliferation data. BrdU incorporation by primary (black) and immortalised (grey) islet MECs during 60 days of culture. Data are expressed as mean value  $\pm$  SD of three experiments. \* $p < 0.05$  vs primary islet MECs

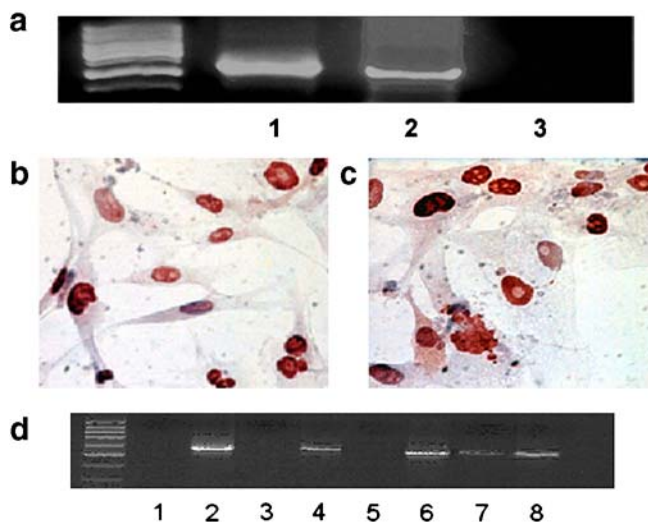
## Statistical analysis

BrdU incorporation, mean values of MFI, the mean percentage of positive cells for surface molecules, and the numbers of adherent PBMCs between primary and immortalised cells were compared using the Mann-Whitney *U*-test. Data were analysed using the SPSS statistical package (SPSS, Chicago, IL, USA), and *p* values less than 0.05 were considered significant.

## Results

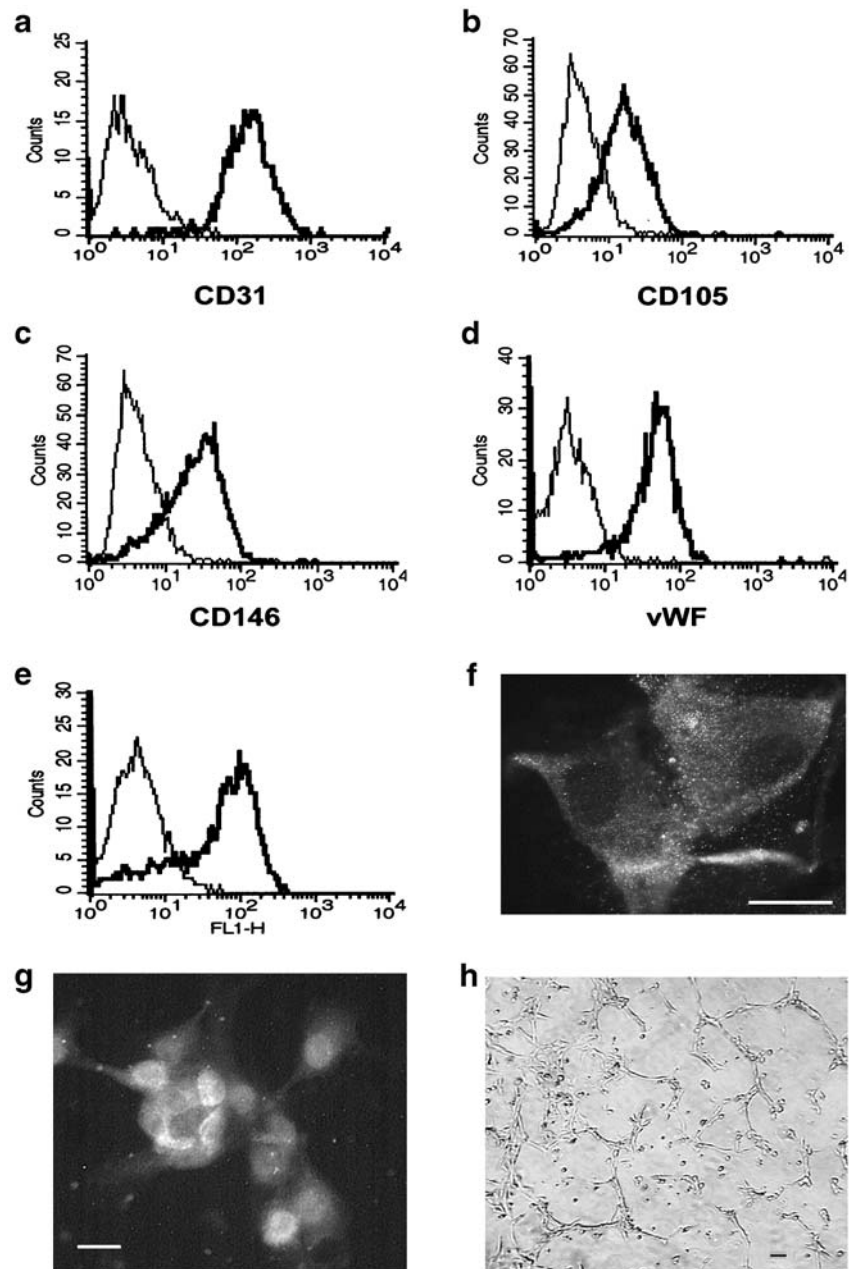
### Primary and immortalised islet endothelial cell culture and phenotype

Isolated primary islet MECs were elongated in shape, and grew in monolayer (Fig. 1a), without the typical cobblestone-like endothelial cell morphology. The morphology of the immortalised counterparts of islet MECs was essentially indistinguishable from that of the primary cells, although they tended to assume a more granular appearance (Fig. 1b). The viability of both cell types was 90 to 96% as detected by trypan blue exclusion.



**Fig. 3** Representative immunocytochemical staining and PCR analysis of large T-Ag expression in immortalised islet MECs. **a** Representative agarose gel resolving RT-PCR products after amplification for full-length SV40 large T-Ag, detected as a band of 2124 bp. Lane 1, immortalised islet MECs; lane 2, vector with cloned large T-Ag; lane 3, primary islet MECs. **b, c** Micrographs of immortalised islet MECs showing that the nuclear staining (red) for SV40 large T-Ag increased in intensity among early (10 passages) (**b**) and late (30 passages) (**c**) subcultures. Original magnification:  $\times 200$ . This was confirmed (**d**) by semi-nested PCR for large T-Ag, detecting the first-round product (575 bp) (lane 1, primary MECs; lane 2, immortalised podocytes; lane 3 early subcultures of immortalised MECs; lane 4, late subcultures) and the second-round product (543 bp) (lane 5 primary MECs; lane 6, immortalised podocytes; lane 7 early subcultures of immortalised MECs; lane 8, late subcultures). The first-round product was negative in early subcultures of immortalised cells, and the intensity of the second-round product increased in late subcultures. Three different experiments were performed with similar results

**Fig. 4** Representative flow cytometric analysis and immunofluorescence micrographs of immortalised islet MECs. Detection of staining for CD31 (a), endoglin (CD105) (b), transmembrane glycoprotein melanoma cell adhesion molecule (CD146) (c), vWF (d), and VE-cadherin (e). Thin lines represent the corresponding isotype control antibodies. Repeated experiments, every three to four subcultures, were performed with similar results. Primary islet MECs exhibited similar patterns. f, g Immunofluorescence enabled nephrin ( $\times 400$ ) and Api ( $\times 200$ ), respectively to be detected in immortalised cells. The appropriate controls with irrelevant immunoglobulins were negative (data not shown). h Immortalised islet MECs cultured on Matrigel for 24 h aligned to form capillary-like structures (representative phase contrast micrograph  $\times 100$ ). Bars: 10  $\mu\text{m}$



By scanning electron microscopy, primary cells appeared flat with a rather smooth surface, and with nuclei and nucleoli visible throughout the even cytoplasm surface.

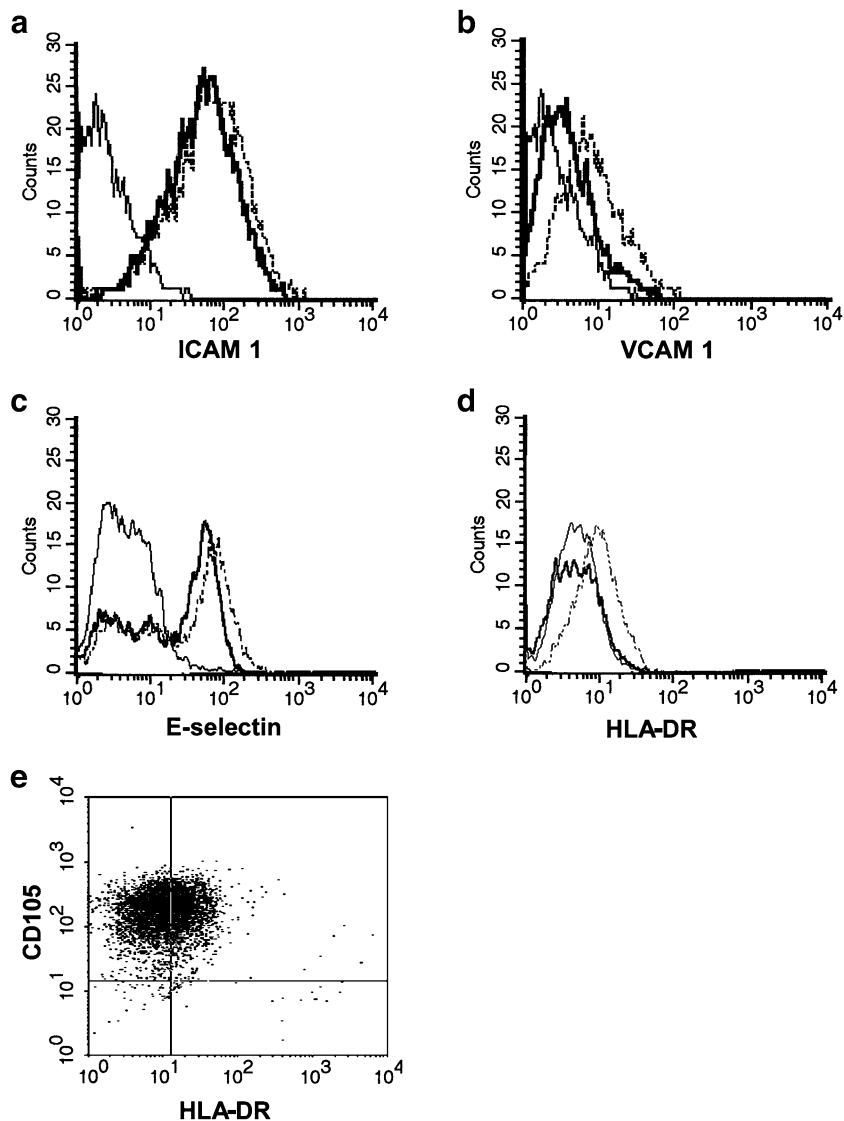
Several fenestrations were detectable (Fig. 1c). TNF treatment stimulated cell retraction, ruffling of the surface, and loss of reciprocal contacts (Fig. 1d). Nuclei and

**Table 1** Mean fluorescence intensity and percentage of cells positive for expression of endothelial cell markers repeatedly assessed during 3 months of culture of immortalised islet MECs

	15 days		1 month		2 months		3 months	
	MFI	Percentage	MFI	Percentage	MFI	Percentage	MFI	Percentage
CD31	114.9	99.8	103	84	55	80	71	86
CD105	18.7	77.3	36	89	23.2	89	25.2	65
CD146	81.9	97.1	85.6	93	52	94.9	81	82
vWF	30	91.7	49	94	31	91.8	22	90
VE-cadherin	30	79.6	22	59	50	89	49	66

MFI mean fluorescence intensity, vWF von Willebrand's factor

**Fig. 5** Representative flow cytometric analysis of adhesion molecules and HLA-DR on immortalised islet MECs. Detection of staining for ICAM1 (a), VCAM1 (b), and E-selectin (c) expression after treatment (dashed line), or not (dark line) with TNF (10 ng/ml for 4 h). **d** IFNG (100 IU/ml) induced expression of HLA-DR molecules (dashed line) in CD105-positive islet MECs, which are represented (e) as signals in the upper right-hand quadrant. Thin lines (a–d) represent the corresponding isotype control antibodies. Four to six experiments were performed with similar results. Primary islet MECs exhibited similar patterns



nucleoli were no longer visible due to the thickening of the cell bodies resulting from the retraction of peripheral cytoplasm. These characteristics were entirely retained by the immortalised cells.

Immortalised islet MECs had the ability to grow to a higher density than primary cells (confluent primary cultures contained mean  $1.5 \times 10^6$  cells/25-cm<sup>2</sup> flask, whereas immortalised cultures contained up to  $2.8 \times 10^6$  cells/25-cm<sup>2</sup> flask) and reached confluence within 5 to

**Table 2** Mean fluorescence intensity and percentage of cells positive for expression of ICAM1, VCAM1 and E-selectin, both basally and after 4 h of TNF treatment

		Primary islet MECs		Immortalised islet MECs	
		MFI	Percentage of positive cells	MFI	Percentage of positive cells
ICAM1	Basal	57.3±33	69.7±14.5	55.8±28.5	79.4±11.2
	+ TNF	70.7±16	78±4	62.2±21.4	74±22.8
VCAM1	Basal	9.7±9	18.9±22	12.9±6	37.7±15.3
	+ TNF	26.1±5.5	51.6±2	21±7	46±15
E-selectin	Basal	16±4	25±7	18±2	31±5
	+ TNF	30±6	32±12	30±2	33±7

Data are expressed as mean value±SD of four to six separate experiments for each flow cytometric analysis. There was no statistical difference between primary and immortalised cells for all adhesion molecules. *MFI* mean fluorescence intensity, *ICAM1* intercellular adhesion molecule-1, *VCAM1* vascular adhesion molecule-1

6 days, whereas primary cultures, after the earlier passages, reached confluence in 9 to 10 days.

The BrdU proliferation assay showed greater DNA synthesis in the immortalised MECs than in primary cells, especially in the late passages (Fig. 2).

Primary islet MECs had a limited life span. Although remaining viable in culture without detaching from the monolayer, they underwent progressive growth arrest and proliferation decreased after approximately seven to eight passages. In contrast, SV40-treated cells had a longer life span than cells from primary cultures. Indeed, to date these cells have been cultured for approximately 30 passages over a span of 150 days.

Immunoperoxidase staining showed that over 90% of the cells of the immortalised islet MEC line were positive for the presence of SV40 large T-Ag within their nuclei. The expression of functional large T protein was confirmed by PCR analysis detecting the complete specific transcript in immortalised MECs as a band of 212 bp (Fig. 3a). We found an increase in the intensity of the nuclear staining among early and late passages of the immortalised cultures (Fig. 3b,c); these differences were confirmed by semi-nested PCR analysis of large T-Ag DNA (Fig. 3d), indicating that during serial subcultures there may be a progressive selection of cells expressing high levels of large T-Ag.

Immortalised islet MECs were not able to form colonies in soft agar, but their growth was serum- and anchorage-dependent and contact-inhibited, thus suggesting a lack of tumorigenic transformation [32]. When implanted subcutaneously in SCID mice, these cells had not induced tumours at 1-month follow up.

Primary and immortalised islet MECs maintained endothelial cell characteristics, as assessed by detection of vWF expression and endothelial-cell associated markers, i.e. endoglin (CD105), CD31, VE-cadherin, and transmembrane glycoprotein melanoma cell adhesion molecule (CD146), by fluorescence assisted cell sorting analysis (Fig. 4a–e). Expression of these molecules was retained when reassessed after every three or four subcultures (Table 1). Moreover, early passages of both cells have been cryopreserved in liquid nitrogen and subsequently thawed, with no major changes in phenotype.

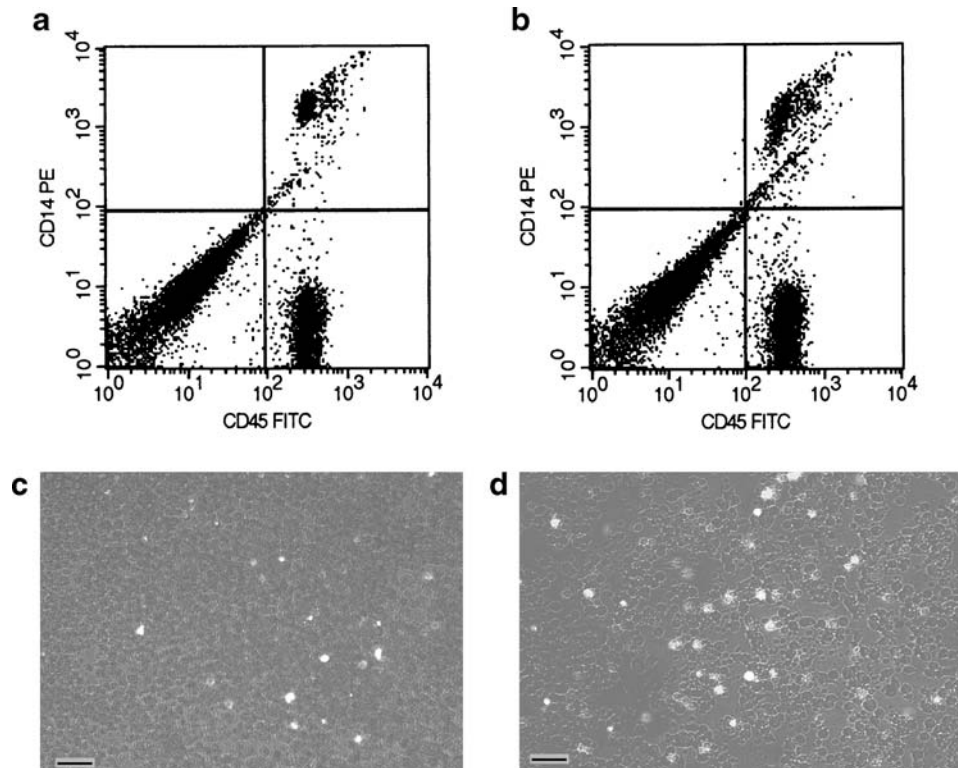
Notably, the specific expression of nephrin and Api persisted in the immortalised counterparts (Fig. 4f,g).

Both primary and immortalised islet MECs formed capillary-like structures when cultured on Matrigel. Both cell types demonstrated cord formation as early as after 2 h and well developed networks of capillary-like structures were present by 24 h (Fig. 4h).

#### Expression of adhesion molecules

After cytofluorimetric analysis primary and immortalised islet MECs showed high expression of ICAM1 (CD54), low basal expression of E-selectin (CD62E) and no detectable expression of VCAM1 (CD106) (Fig. 5a–c). TNF treatment upregulated ICAM1 and E-selectin, and induced neo-expression of VCAM1. Table 2 summarises MFI and the percentage of cells positive for expression of ICAM1 and VCAM1 and E-selectin, both basally and after TNF treatment. There were no statistical differences between primary and immortalised cells for expression of

**Fig. 6** Adhesion of PBMCs on immortalised MEC monolayer. Flow cytometric analysis of harvested adherent PBMCs on untreated (a) and TNF-treated (10 ng/ml for 12 h) (b) MEC monolayers. In the two-parameter cytograms, PBMCs (CD45<sup>+</sup>, CD14<sup>-</sup>) are represented as signals in the *lower right-hand quadrant*, whereas monocytes (CD45<sup>+</sup>, CD14<sup>+</sup>) are represented as signals in the *upper right-hand quadrant*. **c, d** Inverted microscopy detection, in the same experimental conditions, of adherent PKH2-labelled PBMCs on untreated and TNF-treated islet MEC monolayer, respectively. Adherent PBMCs appear as *bright spots*. Bars: 10  $\mu$ m. Primary cell monolayers exhibited similar results





these surface molecules. Neither cell type expressed consistent, detectable CD40 or CD154 (CD40 ligand).

IFNG treatment induced expression of the HLA class II molecules HLA-DR. The magnitude of increase and the time-course of upregulation were similar in primary and immortalised cells (Fig. 5d,e).

### PBMC adhesion and migration

We compared basal and TNF-induced adhesion of PBMCs with primary and immortalised islet MEC monolayers. Flow cytometric analysis of harvested adherent PBMCs and monocytes indicated that treatment with TNF increased adherence to primary cell monolayers (by approximately 40 and 30%, respectively). TNF increased adherence of PBMCs and monocytes to immortalised cell monolayers in a similar way (Fig. 6a,b).

In parallel experiments, digital image analysis of PKH2-labelled PBMCs showed that TNF enhanced PBMC adhesion to primary islet MEC monolayers (unstimulated control  $19.4 \pm 7.4$  PBMCs per microscope field, TNF stimulation  $51.3 \pm 19$ ) and immortalised monolayers (unstimulated control  $19.5 \pm 6.7$ , TNF stimulation  $52.4 \pm 11$ ) (Fig. 6c,d). No statistical difference between primary and immortalised cells was observed.

In PBMC migration experiments, no cells were detectable in the lower chamber without TNF stimulation. Although TNF stimulation increased albumin diffusion, only very few cells were seen in the lower chamber at either 4 or 12 h after stimulation, with the amount of migrating cells reaching approximately 4% of seeded PBMCs at 18 h after stimulation. Results were similar using primary and immortalised MECs.

## Discussion

The present work reports the establishment of the first SV40-immortalised cell line derived from isolated human pancreatic islet microvascular endothelial cells. This work was initiated in an attempt to overcome the drawbacks of working with primary islet endothelial cell cultures, e.g. difficulties in isolation, purification and propagation of large numbers of pure MECs, the same being true of other organs [33–36].

To date, there is only one study of purified human islet MECs [11], and it indicates that the isolation and propagation of MECs from pancreatic islets yields small numbers of cells, and that these cells exhibit a very low proliferation capacity, thus further hampering studies on pancreatic islet microendothelium. Moreover, these islet MECs show distinct morphological and functional characteristics. Notably, they have surface fenestrae [11–13], which are maintained by vascular endothelial cell growth factor and are possibly involved in rapid substance exchange and glucose sensing [12]. These cells express Api [11] and nephrin [19], and also induce insulin gene expression during islet development [14].

In our study, we purified islet MECs that are known to reside within isolated islets and that retain proliferative potential [37, 38]. The purified islet MECs obtained and cultured by us do not exhibit the typical cobble-stone like endothelial phenotype and have surface fenestrations, as shown in vivo [9, 10]; treatment with TNF alters their shape due to cytoskeletal reorganisation [31]. These cells express constitutively high baseline surface levels of the major adhesion molecule ICAM1, and they also express E-selectin and VCAM1 after TNF induction, with a pattern similar to that seen in other microendothelial cell lines, such as human microvascular endothelial cell line-1 [6, 39].

We also characterised the immunological profile of these islet MECs, since it is well established that endothelial cells have a role in inflammatory processes, being able to secrete numerous cytokines and chemokines, and participate in presentation of antigens to T cells [25, 40–42]. Indeed, during insulinitis in type 1 diabetes, endothelial cells surrounding the islets have been shown to assume an activated phenotype [20–24]. IFNG treatment of the islet MECs obtained by us induced expression of HLA class II molecules, whose function is to present antigen to CD4<sup>+</sup> T-cells.

Studies of endothelial–leucocyte interaction, in a static assay, showed enhanced adhesion of blood mononuclear cells induced by TNF, probably mediated by upregulation of the surface molecules involved in this process. Furthermore, the endothelial monolayer formed a barrier to lympho-monocyte transmigration, and although TNF increased permeability, it only minimally enhanced lympho-monocyte passage across the monolayer, indicating that other, additional mediators of inflammation and surface molecule interaction are needed [40].

However, as for all human diploid cells, the primary islet MECs in culture had a limited life span, with progressive reduction of their proliferation capacity until significant senescence and irreversible growth arrest set in [43]. This rendered it even more desirable to establish an immortalised cell line, with the additional aim of circumventing the variability associated with repeated primary isolation and the effects of propagation. However, the process of immortalisation may conceivably result in phenotypic changes, such as reduction in surface antigens and reduced responsiveness to cytokines [8, 44]. By SV40 transfection of early-passage primary cells, we generated an immortalised islet MEC line that has a longer life span than that of primary cells, with a continuous period of vigorous growth. It is known that SV40 large T-Ag can induce immortalisation of the cells by interfering with several pathways related to cell cycle control through the binding and blockade of tumour suppressor proteins such as p53, pRb, p107, and p130/Rb2 [43, 45]. In the present study, over 90% of the cells of the immortalised islet MEC line were positive for nuclear staining specific to SV40 large T-Ag, and molecular evidence indicated that the complete gene sequence was present in cultured cells. These findings unequivocally indicate that replicative senescence of islet MECs was bypassed by the stable expression of SV40 large T-Ag. Although phenotypically homogeneous and stable, the cell line established represents a non-clonal

population of cells, which could eventually be subcloned by limiting dilution.

Cellular immortalisation is known to be a prerequisite of cell transformation [32]. It is known that different cell types are extremely heterogeneous in susceptibility to SV40-mediated transformation [46], which requires the expression of the complete early region (large and small T-Ag) of the virus [47]. The islet MECs in our study expressed the SV40 large T-Ag, and although rapidly expanding, maintained the morphology of the primary cells. Their growth was contact-inhibited, serum-dependent and anchorage-dependent, thus excluding a relevant dedifferentiation process; this could be due to the lack of expression of SV40 small T-Ag, which is considered necessary to generate tumorigenic human cells [47]. Preliminary *in vivo* experiments further suggest the absence of tumorigenic potential. We also demonstrate that these cells retain important biological features characteristic of the parental cells. Our immortalised cells, in fact, behave similarly to primary cells in functional terms, comparing favourably with primary cultures in adhesion molecule expression, support of leucocyte adhesion, and barrier function. Notably, our immortalised cells can still be activated, as shown by TNF stimulation and consequent adhesion molecule upregulation and enhanced adhesion of mononuclear cells, and by INFG induction of HLA class II molecules. They also persistently maintained the endothelial phenotype, thereby serving as a continuously renewable cell line. Notably, they retained expression of Api, an enzyme inhibitor and immune regulator [48, 49], and of nephrin, a transmembrane protein with structural, adhesion and signalling functions, both of which appear to be specific to islet MECs [11, 19].

In conclusion, the immortalised microendothelium described here represents a pancreatic islet endothelial cell line capable of growth and stable phenotype. There is no reason why these cells should not represent their primary counterparts in functional assays, becoming useful tools in dissecting the role of islet microendothelium in the pathophysiology of diabetes mellitus, as well as in developing intervention strategies to achieve optimal engraftment and function of transplanted islets, which is highly dependent on revascularisation [50].

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