E-cadherin Interactions Regulate β -Cell Proliferation in Islet-like Structures

Melanie J. Carvell, Phil J. Marsh, Shanta J. Persaud and Peter M. Jones

Beta Cell Development and Function Group, School of Biomedical and Health Sciences, King's College London

Key Words

Islet of Langerhans • Pancreatic β -cell • E-cadherin • Cell contact • Proliferation

levels do not modulate insulin secretory function but are consistent with a role for ECAD in the regulation of β -cell proliferation.

Copyright © 2007 S. Karger AG, Basel

Abstract

Islet function is dependent on cells within the islet interacting with each other. E-cadherin (ECAD) mediates Ca^{2+} -dependent homophilic cell adhesion between β -cells within islets and has been identified as a tumour suppressor. We generated clones of the MIN6 β -cell line that stably over- (S) and under-express (α S) ECAD. Modified expression of ECAD was confirmed by quantitative RT-PCR, immunoblotting and immunocytochemistry. Preproinsulin mRNA, insulin content and basal rates of insulin secretion were higher in S cells compared to α S and control (V) cells. However, stimulated insulin secretory responses were unaffected by ECAD expression levels. ECAD expression did affect proliferation, with enhanced ECAD expression being associated with reduced proliferation and vice versa. Formation of islet-like structures was associated with a significant reduction in proliferation of V and S cells but not α S cells. These data suggest that ECAD expression

Introduction

E-cadherin (ECAD) is the principal adhesion molecule found in islets of Langerhans where it is believed to play a critical role in regulating islet architecture, as demonstrated by studies showing that disruption of ECAD contacts perturbs islet architecture and affects insulin secretory function [1, 2]. We have previously shown that the insulin-secreting MIN6 cell line forms islet-like structures (termed 'pseudoislets') whose formation is dependent on the expression of ECAD and results in an upregulation of the adhesion molecule and its associated catenin elements [3, 4]. Pseudoislet formation is also associated with enhanced insulin secretory responses to both nutrient and non-nutrient stimuli which is lost upon dispersal of the islet structure [5, 6], suggesting that the gain of function is dependent on the cells being in close contact with each other, and implicating ECAD in modulating insulin secretion.

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2007 S. Karger AG, Basel
1015-8987/07/0205-0617\$23.50/0

Accessible online at:
www.karger.com/cpb

Melanie Carvell,
Beta Cell Development and Function Group
Room 2.4N Hodgkin Building, Guy's Campus
King's College London, London SE1 1UL (GB)
Tel. +442078486279, Fax +442078486280, E-Mail Melanie.Carvell@kcl.ac.uk

ECAD is a member of the type 1 classical cadherin family which mediates Ca^{2+} -dependent cell adhesion in epithelial cells [7]. It is a 120kDa transmembrane protein composed of a highly conserved cytoplasmic domain, a single pass transmembrane domain and five extracellular cadherin-motif subdomains (C1-C5). ECAD molecules form calcium-dependent homophilic interactions through their extracellular domain, and their cytoplasmic domains interact with proteins capable of linking the cadherin molecules to the actin cytoskeleton and cell signalling pathways, including β -catenin or γ -catenin (plakoglobin).

Loss of ECAD expression or function is associated with enhanced cell invasiveness *in vitro* [8-10], or tumour progression *in vivo* [11, 12], suggesting that ECAD may modulate cell proliferation. ECAD engagement influences several intracellular signalling pathways that are involved in the regulation of proliferation, including the Wnt pathway, receptor tyrosine kinases, and Rho GTPase signalling [13]. Thus, β -catenin, in addition to localising to the cell surface and mediating cell adhesion, is also located in the cytoplasm and nucleus where it can function as a mediator of the Wnt signal transduction pathway and thereby play a crucial role in regulating proliferation during development by functioning as a transcriptional co-activator of the TCF/Lef-1 transcription factors and regulating the expression of genes including c-myc [14] and cyclin D1 [15, 16]. In addition, it has been shown that ECAD engagement can promote growth arrest by increasing the expression of cyclin-dependent kinase inhibitors, such as p27^{Kip1}, inducing the dephosphorylation of retinoblastoma protein and reducing cyclin D1 levels [17]. More recently it has been shown that cadherin-mediated adhesion can trigger intracellular signalling events including activation of phosphatidylinositol 3-kinases (PI3-kinases) and the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase pathway [18, 19].

Current evidence suggests multiple functions for ECAD in pancreatic β -cells. In addition to its role as a cell adhesion molecule in maintaining islet structure [1, 3], and a suggested role in the regulation of insulin secretion [2, 3, 5], we have recently demonstrated that the formation of islet-like structures by insulin-secreting cells induced the up-regulation of the expression of the cyclin-dependent kinase inhibitors (CKIs), p21^{Cip1} and p27^{Kip1}, consistent with increased ECAD-mediated cell-cell contact inducing anti-proliferative signals in the cells [20]. The aim of the present study was therefore to over- and under-express ECAD in insulin-secreting cells to determine to what extent ECAD expression influences

β -cell proliferative and secretory function.

Materials and Methods

MIN6 cells were obtained from Dr. Y. Oka and J.-I. Miyazaki (Univ. of Tokyo, Tokyo, Japan). An E-cadherin expression vector, pEM2, was a kind gift from Dr. M. Takeichi (Kyoto University, Kyoto, Japan). DMEM, glutamine, penicillin-streptomycin, gelatin (from bovine skin), PBS, foetal bovine serum, trypsin-EDTA and phorbol myristate acetate (PMA) were from Sigma-Aldrich (Poole, Dorset, U.K.). G418 was obtained from Merck Biosciences (Nottingham, U.K.). RNeasy mini mRNA extraction kit was obtained from Qiagen (Crawley, West Sussex, U.K.). Restriction endonucleases were obtained from Promega (Madison, WI), and pcDNA3.1 was from Stratagene Europe (Amsterdam, The Netherlands). PCR primers were prepared in-house (Molecular Biology Unit, King's College London), and real-time quantitative PCR was performed using a LightCycler rapid thermal cycler system from Roche Diagnostics (Lewes, Sussex, U.K.). Polyacrylamide gels (10%), molecular mass markers, sample buffer, and PAGE buffers were from Invitrogen (Paisley, U.K.). Mouse monoclonal IgG2a ECAD antibody (clone 36) and mouse monoclonal IgG1 β -catenin antibody were from BD Biosciences-Pharmingen (Oxford, U.K.). Mouse monoclonal IgG1 α -catenin antibody and mouse monoclonal IgG2b p21 antibody were from Santa Cruz Biotechnology. Mouse monoclonal IgG1 δ p27 antibody was from Merck Biosciences (Nottingham, UK). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was from Pierce Biotechnology (Rockford, IL) and the Alexa Fluor conjugated secondary antibody was from Invitrogen (Paisley, U.K.). The enhanced chemiluminescence (ECL) detection system and Hyperfilm were from Amersham Pharmacia Biotech International (Buckinghamshire, U.K.). The colorimetric cell proliferation ELISA assay (Roche, Mannheim, Germany) was used in this study to quantify cell proliferation.

Vector construction, transfection of MIN6 cells, and selection of clones

The ECAD coding sequence was excised from the pEM2 vector by a partial EcoR1 digest to yield a 3.1kb fragment which was gel purified and ligated into pcDNA3.1 that had been cut with HindIII and XbaI to enable the ECAD insert to be directionally cloned into the expression vector. The DNA was prepared for transfection by banding plasmid preparations on CsCl according to standard methods [21]. MIN6 cells, grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 2mM glutamine, were electroporated (2kV/cm, 3 μ F) with PvuI-linearised ECAD sense or antisense vector ('S' and ' α S', respectively), or with linearised pcDNA3.1 to which ECAD cDNA had not been ligated (empty vector, 'V'). Neo-resistant transfected cells were selected by growth in medium supplemented with 0.8mg/ml G418. Colonies of resistant MIN6 cells were expanded for analysis and functional studies.

Cell culture and pseudoislet formation

MIN6 cells were maintained at 37°C/5% CO₂ in DMEM supplemented with FBS, penicillin/streptomycin, glutamine and G418, as described above. The medium was changed every 3 days and the monolayers were passaged or used for experiments when 70% confluent. MIN6 pseudoislets were generated by culturing MIN6 cells for 7 days on tissue culture flasks that had been precoated with gelatin, as described previously [22].

Insulin secretion

Basal rates of insulin secretion were assessed by static incubation, where groups of 50,000 cells were incubated for 1h in the presence of a physiological salt solution supplemented with 2mM glucose and insulin secretion was measured by radioimmunoassay, as described. Dynamic insulin release was assessed using a multichamber perfusion system at 37°C in a temperature-controlled environment. Basal secretion was assessed by perfusing age- and passage-matched pseudoislets with 2mM glucose, insulin secretion was stimulated by the addition of 20mM glucose and then further enhanced by the addition of PMA to the perfusate. Samples were collected at 2-minute intervals and insulin secretion was measured by radioimmunoassay [24].

E-cadherin and preproinsulin mRNA expression

Messenger RNA was isolated from control and ECAD over- and under-expressing MIN6 cells using the RNeasy Mini Kit according to the manufacturer's instructions, mRNA was quantified using a Nanodrop spectrometer (NanoDrop, Rockland, ME) and cDNA was synthesised as described [25]. Mouse ECAD forward and reverse PCR primers were as follows: forward, 5'-CGT GAT GAA GGT CTC AGC C-3'; reverse, 5'-ATG GGG GCT TCA TTC AC-3' (amplifies a fragment of 616bp). Forward and reverse actin PCR primers were as follows: forward: 5'-ACG GCC AAG TCA TCA CTA TTG-3'; reverse: 5'-AGC CAC CGA TCC ACA CAG A-3', the predicted size of the actin PCR product was 300bp. ECAD standards ranging from 10 copies to 10⁹ copies DNA were prepared as described [26]. Real-time PCR amplification was performed using a LightCycler rapid thermal cycler system. Reactions were performed in a 10µl volume containing nucleotides, Taq DNA polymerase, and buffer (all included in the LightCycler FastStart Reaction Mix SYBR Green I); template cDNA; 1.5mM MgCl₂ and 0.5µM primers. All PCR protocols included an initial 10min denaturation step and each cycle subsequently included a 95°C denaturation for 0s, annealing for 10s at 58°C (actin) or 56°C (ECAD), and a 72°C extension phase for 14s (actin) or 25s (ECAD). Fluorescence measurements were taken at 83°C (actin) or 85°C (ECAD) for 3s to eliminate fluorescence from primer-dimer formation. The amplification products of both primer pairs were subjected to melting point analyses and subsequent gel electrophoresis to ensure specificity of amplification.

Western blotting

Monolayer MIN6 cells were detached with 0.02% EDTA solution to avoid proteolytic degradation of cell surface proteins. Extracts of G418-resistant β-cell clones were prepared by sonication in a lysis buffer [27], and total protein content

was determined by the Bradford method [28] using bovine serum albumin as standard. Equivalent amounts of total protein from each sample were separated by gel electrophoresis on NuPAGE gels and the resolved proteins were electrotransferred onto polyvinylidene difluoride membranes [29]. Membranes were probed with the appropriate primary (0.1µg/ml–1.0µg/ml) and secondary HRP-conjugated antibodies. Antibody binding was visualised using an ECL system, and quantified by densitometric scanning of developed film using a SynGene Bio imaging system.

Immunofluorescence

MIN6 cells were seeded onto APES-coated coverslips and cultured for 3-5 days. Immunofluorescent detection of protein expression was performed after fixing the cells using 4% (w/v) paraformaldehyde. Cells were incubated overnight at 4°C with a monoclonal anti-ECAD mouse primary antibody (1:250 dilution) in 5% milk (in PBS/0.1% triton) followed by an Alexa Fluor conjugated secondary antibody (1:200 dilution in PBS / 0.3% triton) at RT. Immunofluorescence was visualised under a fluorescent microscope.

Cell Proliferation

DNA synthesis as a marker of cell proliferation was assessed by measuring the incorporation of 5-bromo-2'-deoxyuridine (BrdU) using a commercially available kit, essentially according to the manufacturer's instructions. Empty vector, over- and under-expressing MIN6 cells were seeded into 96-well microtitre plates at a density of 20,000 cells/well, and left to adhere overnight at 37°C/5% CO₂, and the cells were then processed as described previously [30]. Where proliferation was compared between monolayer and pseudoislet populations, the method was followed as previously described [31]. BrdU incorporation was expressed as absorbance units per 2x10⁴ cells after correction for cell-free blanks.

Results

Experimental manipulation of ECAD expression in MIN6 cells

The transfection and clonal selection of MIN6 cells generated populations of cells with modifications in the expression of ECAD. Initial screening of 7 separate clones using real-time quantitative PCR measurements of ECAD mRNA normalised against β-actin mRNA enabled the selection of individual clones. Analysis of the selected clones revealed greatly increased expression of ECAD mRNA in transfectants harbouring the ECAD expression vector (S), compared to those transfected with vector alone (V) (S, 317±91% increase over V; p<0.05, (Figure 1). Conversely, cells transfected with the vector in the antisense orientation (αS), showed significantly reduced expression of ECAD mRNA (αS, 52±13% content of V; p<0.05). These changes in mRNA were

Fig. 1. Experimental manipulation of ECAD expression in MIN6 cells: ECAD mRNA expression: MIN6 cells were transfected with pcDNA3.1 vector alone (V), or with pcDNA3.1 vector housing the ECAD coding sequence in the sense (S) or antisense (α S) orientation, and selected by maintenance in medium supplemented with G418. ECAD mRNA levels were significantly (* $p < 0.05$) increased in S cells and decreased in α S cells when compared to V cells. Bars show mean $\pm$ SEM, $n=7$.

reflected by changes in the levels of immunoreactive ECAD protein, as shown in Figure 2A. Immunoblotting measurements demonstrated increased ECAD expression in S transfectants compared to protein-matched samples prepared from V control cells (Figure 2A, upper panel). Densitometric analysis of immunoblots of sub-maximal amounts S cell extract (Figure 2A upper panel, lanes 3,4) demonstrated a 249% increase in ECAD protein content, consistent with the approximately 3-fold increase in ECAD mRNA expression (Figure 1). Similarly, analysis of ECAD immunoblots using extracts of α S cells showed a 63% reduction in ECAD protein content, consistent with the approximately 50% reduction in ECAD mRNA expression in these cells. Concomitant changes in the expression of β -catenin were not detected (S cells, 108% control; α S cells, 137% control, Figure 2B), although α -catenin expression was downregulated in under-expressing transfectants compared to their protein-matched control and S counterparts (S cells, 92% control; α S cells 39% control, Figure 2C). Similar results were obtained when ECAD immunoreactivity was assessed by immunocytochemistry, as shown in Figure 3. Thus ECAD immunofluorescence was detectable at low levels in control (V) cells, whereas the ECAD over-expressing (S) cells demonstrated a greatly-enhanced fluorescent signal which was principally localised around the periphery of cells within the clusters. Neither over-expression nor under-expression of ECAD was associated with any detectable changes in cell morphology as assessed by light microscopy when the cells were

Fig. 2. Immunoblot analysis of ECAD and catenin expression: [A] Immunoblot analysis using 20 μ g and 10 μ g of MIN6 cell protein extracts demonstrated that ECAD expression was markedly increased in ECAD over-expressing MIN6 cell lysates (S) (upper panel) and decreased in ECAD under-expressing MIN6 cell lysates (α S) (lower panel) compared to protein-matched empty vector control (V) cell lysates. [B] Immunoblot analysis using 40 μ g of MIN6 cell protein extracts showed that no changes in β -catenin expression were detectable between the transfectants. [C] Immunoblot analysis demonstrated that α -catenin expression was reduced in α S MIN6 cell lysates compared to protein-matched (40 μ g) V and S cell lysates.

maintained as monolayer cultures on tissue culture plastic. All three cell types formed islet-like structures (pseudoislets) when maintained in culture on a gelatin substrate and there were no detectable differences in the rate of pseudoislet formation. Pseudoislets formed

Fig. 3. ECAD expression and pseudoislet formation: The figure shows fluorescence immuno-
cytochemical staining for ECAD
expression in clusters of
monolayer MIN6 cells grown on
tissue culture plastic. Low levels
of ECAD immunoreactivity were
detected in control (V) cells (left
panel), but there were much more
pronounced levels of ECAD
expression in S cells (right panel),
with ECAD immunoreactivity
being localised around the
periphery of the cells forming the
clusters. Bars show 50 μ m scale.

Fig. 4. ECAD expression and proliferation in MIN6 monolayer
cells: [A] Expression of the cyclin-dependent kinase inhibitor
p27^{Kip1} expression was downregulated in α S cell lysates
compared to protein-matched (20 μ g) V and S cell lysates. [B]
ECAD over-expressing S cells showed a reduced rate of cell
proliferation as assessed by BrdU incorporation when
compared to control V cells. BrdU incorporation is expressed
as absorbance units per 2 $\times$ 10⁴ cells after correction for cell-free
blanks (points show means $\pm$ SEM, n=10, *** p<0.0001). [C] In
contrast, ECAD under-expressing α S cells showed a
significantly increased rate of cell proliferation as assessed by
BrdU incorporation when compared to control V cells (points
show means $\pm$ SEM, n=12, *** p<0.0001).

from S cells consistently appeared to be more compact and dense, and had a notably smoother outer surface when compared to pseudoislets formed from control V cells. Pseudoislets formed from ECAD under-expressing α S cells tended to have a more diffuse appearance. Pseudoislets formed from control MIN6 cells do not show detectable levels of central necrosis within 7 to 9 days [4], and this was not affected by the over- or under-expression of ECAD.

Effects of ECAD expression on insulin expression and secretion

Levels of (pre)proinsulin (PPI) mRNA were increased by 70% in ECAD-overexpressing S cells, and reduced by 14% in α S cells, compared to V cells. Furthermore, S cells contained significantly higher levels of immunoreactive insulin than V cells (V, 0.11 $\pm$ 0.05 pg/cell; S, 0.19 $\pm$ 0.03; α S 0.09 $\pm$ 0.03, n=4, P<0.05 S vs V). In accordance with the differences in insulin content,

ECAD-overexpressing cells demonstrated higher basal (2mM glucose) rates of insulin secretion than either control (V) cells or ECAD under-expressing (α S) cells (V, 50 $\pm$ 5 fg/cell/h, mean $\pm$ SEM, n=9; S, 120 $\pm$ 16, α S, 70 $\pm$ 5, p<0.05 S vs V). However, when expressed relative to

Fig. 5. ECAD expression and proliferation in MIN6 pseudoislets: Configuring control MIN6 cells (V) or ECAD over-expressing cells (S) as pseudoislets caused a marked reduction in proliferation, as assessed by BrdU incorporation. In contrast, forming pseudoislets from α S cells had no detectable effect on the rate of cell proliferation. To allow direct comparison of BrdU incorporation into pseudoislet cells between the three populations of transfectants, results are expressed as a percentage of incorporation into the appropriate cell type when configured as monolayers. Bars show means $\pm$ SEM, $n=11$, *** $p<0.0001$).

the appropriate basal rate of insulin secretion neither over-expression nor under-expression of ECAD had any detectable effect on stimulated insulin secretory responses whether the MIN6 cells were configured as monolayers or as pseudoislets. Thus, the responses of S or α S pseudoislets to a stimulatory concentration (20mM) of glucose were not significantly different from those of pseudoislets formed from control (V) cells (S, $110\pm 3\%$ control V cells; α S, $108\pm 2\%$, means $\pm$ SEM, $n=5$, $p>0.2$), and similar results were obtained when secretion was stimulated using 500nM of the protein kinase C activator, PMA (S, $93\pm 18\%$ control V cells; α S $92\pm 13\%$, means $\pm$ SEM, $n=5$, $P>0.2$).

Effects of ECAD expression on cell proliferation

Manipulating ECAD expression had significant effects on the proliferative capacity of MIN6 cells maintained in monolayer culture, as shown in Figure 4. Immunoblot measurements of the cyclin-dependent kinase inhibitor, p27^{Kip1} showed a marked reduction of expression in the ECAD under-expressing α S cells compared to either empty vector control cells (V) or ECAD over-expressing S cells (S cells, 140% control; α S cells, 50% control, Figure 4A), consistent with a reduced number of α S cells entering G1 cell cycle arrest. This was confirmed

by direct measurements of DNA synthesis as assessed by BrdU incorporation (Figure 4B, C). Thus, ECAD over-expressing S cells showed a significant reduction in the rate of BrdU incorporation when compared to control V cells (Figure 4B), whereas BrdU incorporation was significantly enhanced in ECAD under-expressing cells compared to controls (Figure 4C). We have previously reported that configuring unmodified MIN6 cells as pseudoislets resulted in changes in the expression of p27^{Kip1} and p21^{Cip1} consistent with reduced proliferation [32]. Figure 5 shows that pseudoislet formation was associated with a significant reduction in proliferation as assessed by BrdU incorporation for both control V cells and ECAD over-expressing S cells. In contrast, configuring ECAD under-expressing α S cells as pseudoislets had no detectable effect on the rate of proliferation (Figure 5).

Discussion

Primary islets of Langerhans are complex functional units which play a crucial role in regulating glucose homeostasis and their function is known to be dependent on the islet cells being in close contact with each other: islet dispersal results in poor secretory responsiveness which can be restored upon reaggregation of the islet cells [33-35]. ECAD is the principal adhesion molecule found in the insulin-secreting islet β -cells and in addition to mediating cell adhesion, it is now also recognised as playing a role in regulating signal transduction events within the cell [36-39]. Here we have shown that following stable transfection of insulin-secreting MIN6 cells with ECAD sense or antisense expression vectors, ECAD was over-expressed both at the mRNA and protein level in MIN6 cells harbouring the ECAD sense expression vector, while mRNA and protein expression were downregulated in cells transfected with the ECAD antisense expression vector, and maintenance of the altered phenotypes was confirmed by assessing mRNA and protein expression for over a year post-transfection. When the transfectant cells were induced to form islet-like structures, their morphology was consistent with the levels of ECAD expression, with S pseudoislets showing a more compacted morphology compared to their V and α S counterparts.

Numerous studies have demonstrated that cell-cell communication within islets is required for normal insulin secretory responses, and our studies using MIN6 cells configured as islet-like structures [3-5] suggest that

homotypic interactions between β -cells are an important part of this process. However, the role of ECAD in the maintenance of insulin secretion is uncertain. Transgenic mice carrying β -cell targeted mutations in hepatocyte nuclear factor 1 α (HNF1 α) showed reduced expression of ECAD in β -cells and impaired glucose-induced insulin secretion *in vivo* [2], consistent with an involvement of ECAD in the regulation of insulin secretion. Furthermore, disrupting islet ECAD function *in vitro* with an anti-ECAD antibody attenuated glucose-induced elevations in β -cell cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_i$) and insulin secretion [2], suggesting a causal link between ECAD expression and secretory function. However, exposing primary islets [2] or pseudoislets [3] to anti-ECAD antibodies also resulted in a disruption of islet structure, with a gradual dispersal of the compact islet anatomy accompanied by the release of cells and cell clusters from the islet structure, so it is difficult to distinguish between direct effects of ECAD blockade on the secretory process and indirect effects secondary to the loss of islet architecture. Our previous demonstration that a similar disruption of pseudoislet structure by methods unrelated to ECAD function generated a very similar phenotype, with marked reductions in glucose-induced elevations in $[\text{Ca}^{2+}]_i$ [40] and insulin secretion [5], suggest that the disruption of islet architecture may be the more important mechanism. Similarly, clonal selection of MIN6 cells that respond well to secretory stimuli revealed elevated (3–4 fold) levels of ECAD expression [41]. However, the enhanced secretory responses were dependent on cell density, suggesting that the ECAD was acting indirectly to increase cell-cell aggregation and thus increase insulin secretion, rather than having a direct effect on the secretory capacity of individual cells. The results of the present study, using direct manipulation of ECAD expression, do not support an important role for ECAD in the regulation of insulin secretion. Thus, ECAD overexpression caused a significant increase in insulin content in S MIN6 cells and this was consistent with their significantly higher basal rates of insulin secretion, perhaps suggesting that ECAD overexpression affected the capacity of the cells to store insulin, consistent with ECAD being associated with a fully differentiated phenotype [42]. However, the raised insulin content did not affect the capacity of S cells to respond to nutrient and non-nutrient stimuli with enhanced insulin secretion. When insulin secretion was expressed relative to the appropriate basal levels of secretion, S cells showed secretory profiles that were not significantly different from those obtained with control or α S cells. These results are in accordance with a previous study

using MIN6 cells in which down-regulation of the gap-junction protein connexin 36 (Cx36) resulted in the attenuation of insulin secretory responses and a loss of ECAD expression [43]. The subsequent over-expression of Cx36 in these cells rescued the insulin secretory responses, whereas normalisation of ECAD expression alone did not affect insulin secretion [44]. Therefore, observations made using different experimental approaches are consistent in suggesting that β -cell ECAD does not play an important role in modulating insulin secretory function.

In contrast, our measurements of DNA synthesis and the expression of cell-cycle regulators support an important role for ECAD in β -cell proliferation. ECAD expression is frequently lost in human epithelial cancers and has therefore been identified as a tumour suppressor [45–47]. In accordance with this, loss of ECAD expression in β -cell tumours *in vivo* is associated with tumour progression and metastases [10], while the islet hyperplasia induced by the β -cell-targeted deletion of the *Men1* tumour-suppressing gene is accompanied by reduced expression of ECAD [48]. Here we show that underexpressing ECAD resulted in reduced levels of the cyclin-dependent kinase inhibitor p27^{Kip1}, which is involved in G1 phase arrest. Furthermore, direct measurements of proliferation by assessing the rate of BrdU incorporation demonstrated that S MIN6 cells were less proliferative than the V controls, while the α S cells proliferated at a faster rate than controls, suggesting that the level of ECAD expression may indeed affect the proliferative capacity of the cells. The increased insulin content detected in the S cells is consistent with increased differentiation as a consequence of reduced proliferation, as has been reported previously using the β TC-tet cell line in which suppression of β TC cell proliferation resulted in increased insulin content [49].

Previously, we have shown that configuring MIN6 cells as pseudoislets resulted in increased E-cadherin expression along with an upregulation of both β - and α -catenin [50], suggesting that upon pseudoislet formation the cells are induced to upregulate all components of the adhesion complex to account for the increased level of adhesion required in a three-dimensional configuration. The changes in ECAD and catenin expression were associated with changes in proliferative markers, such as p21^{Cip1} and p27^{Kip1}, consistent with the generation of antiproliferative signals [51]. The present results support a role for ECAD in the regulation of β -cell proliferation in response to direct cell-cell contact. When V and S transfectants were configured as pseudoislets their

proliferation rates were significantly reduced compared to the rates observed with their equivalent monolayer cells. In contrast, α S cells and pseudoislets proliferated at the same rate indicating that the mechanism by which MIN6 cells suppress proliferation in the pseudoislet conformation is lost when ECAD is under-expressed in MIN6 cells. Although MIN6 cells are an SV40 TAG transformed cell line in which the cell cycle is deregulated, our results and others using this cell line [52, 53] demonstrate that a proportion of their proliferation is still regulatable. Thus, the changes in expression of proliferative markers and BrdU incorporation suggest that antiproliferative signals generated by homotypic ECAD interactions may play an important role in regulating β -cell proliferation. The regulation of β -cell proliferation by ECAD interactions is consistent with our previous findings of enhanced rate of apoptosis in pseudoislets as a mechanism of maintaining pseudoislet size [54]. In the pseudoislet configuration the close contact between cells will enhance ECAD-mediated interactions, whereas these interactions will be reduced or absent when cells are maintained in the monolayer configuration.

One intracellular mechanism through which ECAD may regulate proliferation is via β -catenin. This is a multifunctional protein which, in addition to mediating cell adhesion with ECAD, can also play an important role in regulating cell growth and differentiation by forming bipartite transcription factors with the lymphoid enhancer-binding factor (LEF)-1/T cell factor (TCF) family where the DNA-binding and transactivating functions are contributed by TCFs and β -catenin, respectively [55, 56]. c-Myc [57] and cyclin D1 [58, 59], have been identified as targets of the β -catenin–LEF-1/TCF signalling pathway in colon cancer cells. It has been proposed that the proportion of cytosolic to membrane-associated β -catenin levels plays a critical role in regulating cellular responses to extracellular signals for cell adhesion and cell proliferation, and ECAD can modulate β -catenin signalling by recruiting free cytoplasmic β -catenin to the plasma membrane [60]. In the present study no changes in β -catenin expression were detected by immunoblot

analysis, which may suggest that β -catenin is abundantly expressed within the cell as a multifunctional protein. Interestingly, α -catenin expression was downregulated in α S cells, in accordance with a previous study in prostate cancer cells where loss of E-cadherin expression was associated with a concomitant reduction in α -catenin expression [61, 62]. Furthermore, it has been demonstrated that exogenous expression of α -catenin in α -catenin-deficient cells restored cell adhesion and was accompanied by a retardation of cell growth [63], while targeted deletion of α -catenin in either the skin or in neural progenitor cells of the cerebral cortex resulted in hyperproliferation [64] and it has been shown that changes in α -catenin levels can affect p27^{Kip1} expression levels [65]. In addition, α -catenin may modulate cell proliferation indirectly by associating with β -catenin and thereby inhibiting it from functioning as a transcriptional coactivator [66]. Thus, in addition to mediating cell adhesion, α -catenin also exists in cadherin-independent pools that may play a role in regulating cell proliferation; its reduced expression in α S cells supports the findings that these cells expressed lower levels of p27^{Kip1} and were more proliferative than the controls.

In summary, the present study suggests that homotypic ECAD interactions between neighbouring β -cells in islet-like structures are not involved in regulating insulin secretion but may act to limit β -cell proliferation during islet formation. ECAD-mediated interactions between β -cells may therefore play an important role in the expansion of islet/ β -cell mass during fetal development, or in the adult pancreas in response to the metabolic demands of pregnancy or obesity [67].

Acknowledgement

This work was supported by a grant from the European Foundation for the Study of Diabetes (EFSD) under the EFSD/JDRF/Novo Nordisk Type 1 Diabetes Research Programme.

References

- Dahl U, Sjodin A, Semb H: Cadherins regulate aggregation of pancreatic beta-cells in vivo. *Development* 1996;122(9):2895-2902.
- Yamagata K, Nanno T, Moriwaki M, Ihara A, Iizuka K, Yang Q, Satoh T, Li M, Uenaka R, Okita K, Iwahashi H, Zhu Q, Cao Y, Imagawa A, Tochino Y, Hanafusa T, Miyagawa J, Matsuzawa: Overexpression of dominant-negative mutant hepatocyte nuclear factor-1 alpha in pancreatic beta-cells causes abnormal islet architecture with decreased expression of E-cadherin, reduced beta-cell proliferation, and diabetes. *Diabetes* 2002;51(1):114-123.

- 3 Hauge-Evans AC, Squires PE, Persaud SJ, Jones PM: Pancreatic beta-cell-to-beta-cell interactions are required for integrated responses to nutrient stimuli: enhanced Ca²⁺ and insulin secretory responses of MIN6 pseudoislets. *Diabetes* 1999;48(7):1402-1408.
- 4 Luther MJ, Davies E, Muller D, Harrison M, Bone AJ, Persaud SJ, Jones PM: Cell-to-cell contact influences proliferative marker expression and apoptosis in MIN6 cells grown in islet-like structures. *Am J Physiol Endocrinol Metab* 2005;288(3):E502-E509.
- 5 Hauge-Evans AC, Squires PE, Persaud SJ, Jones PM: Pancreatic beta-cell-to-beta-cell interactions are required for integrated responses to nutrient stimuli: enhanced Ca²⁺ and insulin secretory responses of MIN6 pseudoislets. *Diabetes* 1999;48(7):1402-1408.
- 6 Luther MJ, Hauge-Evans A, Souza KL, Jorns A, Lenzen S, Persaud SJ, Jones PM: MIN6 beta-cell-beta-cell interactions influence insulin secretory responses to nutrients and non-nutrients. *Biochem Biophys Res Commun* 2006;343(1):99-104.
- 7 Gumbiner BM: Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* 1996;84(3):345-357.
- 8 Birchmeier W, Behrens J: Cadherin expression in carcinomas: role in the formation of cell junctions and the prevention of invasiveness. *Biochim Biophys Acta* 1994;1198(1):11-26.
- 9 Simard D, Nabi IR: Inverse relation of autocrine motility factor receptor and E-cadherin expression following MDCK epithelial cell transformation. *Biochem Biophys Res Commun* 1996;219(1):122-127.
- 10 Vermeulen SJ, Bruyneel EA, Bracke ME, De Bruyne GK, Vennkens KM, Vleminckx KL, Berx GJ, van Roy FM, Mareel MM: Transition from the noninvasive to the invasive phenotype and loss of alpha-catenin in human colon cancer cells. *Cancer Res* 1995;55(4):4722-4728.
- 11 Perl AK, Wilgenbus P, Dahl U, Semb H, Christofori G: A causal role for E-cadherin in the transition from adenoma to carcinoma. *Nature* 1998;392(6672):190-193.
- 12 Toyoyama H, Nuruki K, Ogawa H, Yanagi M, Matsumoto H, Nishijima H, Shimotakahara T, Aikou T, Ozawa M: The reduced expression of e-cadherin, alpha-catenin and gamma-catenin but not beta-catenin in human lung cancer. *Oncol Rep* 1999;6(1):81-85.
- 13 Wheelock MJ, Johnson KR: Cadherin-mediated cellular signaling. *Curr Opin Cell Biol* 2003;15(5):509-514.
- 14 He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW: Identification of c-MYC as a target of the APC pathway. *Science* 1998;281(5382):1509-1512.
- 15 Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R, Ben-Ze'ev A: The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. *Proc Natl Acad Sci U S A* 1999;96(10):5522-5527.
- 16 Tetsu O, McCormick F: Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* 1999;398(6726):422-426.
- 17 St Croix B, Sheehan C, Rak JW, Florenes VA, Slingerland JM, Kerbel RS: E-Cadherin-dependent growth suppression is mediated by the cyclin-dependent kinase inhibitor p27(KIP1). *J Cell Biol* 1998;142(2):557-571.
- 18 Pece S, Gutkind JS: Signaling from E-cadherins to the MAPK pathway by the recruitment and activation of epidermal growth factor receptors upon cell-cell contact formation. *J Biol Chem* 2000;275(52):41227-41233.
- 19 Pece S, Chiariello M, Murga C, Gutkind JS: Activation of the protein kinase Akt/PKB by the formation of E-cadherin-mediated cell-cell junctions. Evidence for the association of phosphatidylinositol 3-kinase with the E-cadherin adhesion complex. *J Biol Chem* 1999;274(27):19347-19351.
- 20 Luther MJ, Davies E, Muller D, Harrison M, Bone AJ, Persaud SJ, Jones PM: Cell-to-cell contact influences proliferative marker expression and apoptosis in MIN6 cells grown in islet-like structures. *Am J Physiol Endocrinol Metab* 2005;288(3):E502-E509.
- 21 Radloff R, Bauer W, Vinograd J: A dye-buoyant-density method for the detection and isolation of closed circular duplex DNA: the closed circular DNA in HeLa cells. *Proc Natl Acad Sci U S A* 1967;57(5):1514-1521.
- 22 Hauge-Evans AC, Squires PE, Persaud SJ, Jones PM: Pancreatic beta-cell-to-beta-cell interactions are required for integrated responses to nutrient stimuli: enhanced Ca²⁺ and insulin secretory responses of MIN6 pseudoislets. *Diabetes* 1999;48(7):1402-1408.
- 23 Luther MJ, Hauge-Evans A, Souza KL, Jorns A, Lenzen S, Persaud SJ, Jones PM: MIN6 beta-cell-beta-cell interactions influence insulin secretory responses to nutrients and non-nutrients. *Biochem Biophys Res Commun* 2006;343(1):99-104.
- 24 Luther MJ, Hauge-Evans A, Souza KL, Jorns A, Lenzen S, Persaud SJ, Jones PM: MIN6 beta-cell-beta-cell interactions influence insulin secretory responses to nutrients and non-nutrients. *Biochem Biophys Res Commun* 2006;343(1):99-104.
- 25 Persaud SJ, Roderigo-Milne HM, Squires PE, Sugden D, Wheeler-Jones CP, Marsh PJ, Belin VD, Luther MJ, Jones PM: A key role for beta-cell cytosolic phospholipase A(2) in the maintenance of insulin stores but not in the initiation of insulin secretion. *Diabetes* 2002;51(1):98-104.
- 26 Persaud SJ, Roderigo-Milne HM, Squires PE, Sugden D, Wheeler-Jones CP, Marsh PJ, Belin VD, Luther MJ, Jones PM: A key role for beta-cell cytosolic phospholipase A(2) in the maintenance of insulin stores but not in the initiation of insulin secretion. *Diabetes* 2002;51(1):98-104.
- 27 Luther MJ, Davies E, Muller D, Harrison M, Bone AJ, Persaud SJ, Jones PM: Cell-to-cell contact influences proliferative marker expression and apoptosis in MIN6 cells grown in islet-like structures. *Am J Physiol Endocrinol Metab* 2005;288(3):E502-E509.
- 28 Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248-254.
- 29 Luther MJ, Davies E, Muller D, Harrison M, Bone AJ, Persaud SJ, Jones PM: Cell-to-cell contact influences proliferative marker expression and apoptosis in MIN6 cells grown in islet-like structures. *Am J Physiol Endocrinol Metab* 2005;288(3):E502-E509.
- 30 Luther MJ, Davies E, Muller D, Harrison M, Bone AJ, Persaud SJ, Jones PM: Cell-to-cell contact influences proliferative marker expression and apoptosis in MIN6 cells grown in islet-like structures. *Am J Physiol Endocrinol Metab* 2005;288(3):E502-E509.
- 31 Luther MJ, Davies E, Muller D, Harrison M, Bone AJ, Persaud SJ, Jones PM: Cell-to-cell contact influences proliferative marker expression and apoptosis in MIN6 cells grown in islet-like structures. *Am J Physiol Endocrinol Metab* 2005;288(3):E502-E509.
- 32 Luther MJ, Davies E, Muller D, Harrison M, Bone AJ, Persaud SJ, Jones PM: Cell-to-cell contact influences proliferative marker expression and apoptosis in MIN6 cells grown in islet-like structures. *Am J Physiol Endocrinol Metab* 2005;288(3):E502-E509.

- 33 Halban PA, Wollheim CB, Blondel B, Meda P, Niesor EN, Mintz DH: The possible importance of contact between pancreatic islet cells for the control of insulin release. *Endocrinology* 1982;111(1):86-94.
- 34 Hopcroft DW, Mason DR, Scott RS: Structure-function relationships in pancreatic islets: support for intraislet modulation of insulin secretion. *Endocrinology* 1985;117(5):2073-2080.
- 35 Pipeleers D, in't Veld PI, Maes E, Van De WM: Glucose-induced insulin release depends on functional cooperation between islet cells. *Proc Natl Acad Sci U S A* 1982;79(23):7322-7325.
- 36 Behrens J: Cadherins and catenins: role in signal transduction and tumor progression. *Cancer Metastasis Rev* 1999;18(1):15-30.
- 37 Brembeck FH, Rosario M, Birchmeier W: Balancing cell adhesion and Wnt signaling, the key role of beta-catenin. *Curr Opin Genet Dev* 2006;16(1):51-59.
- 38 Pece S, Chiariello M, Murga C, Gutkind JS: Activation of the protein kinase Akt/ PKB by the formation of E-cadherin-mediated cell-cell junctions. Evidence for the association of phosphatidylinositol 3-kinase with the E-cadherin adhesion complex. *J Biol Chem* 1999;274(27):19347-19351.
- 39 Pece S, Gutkind JS: Signaling from E-cadherins to the MAPK pathway by the recruitment and activation of epidermal growth factor receptors upon cell-cell contact formation. *J Biol Chem* 2000;275(52):41227-41233.
- 40 Hauge-Evans AC, Squires PE, Persaud SJ, Jones PM: Pancreatic beta-cell-to-beta-cell interactions are required for integrated responses to nutrient stimuli: enhanced Ca²⁺ and insulin secretory responses of MIN6 pseudoislets. *Diabetes* 1999;48(7):1402-1408.
- 41 Lilla V, Webb G, Rickenbach K, Maturana A, Steiner DF, Halban PA, Irminger JC: Differential gene expression in well-regulated and dysregulated pancreatic beta-cell (MIN6) sublines. *Endocrinology* 2003;144(4):1368-1379.
- 42 Gumbiner BM: Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* 1996;84(3):345-357.
- 43 Calabrese A, Caton D, Meda P: Differentiating the effects of Cx36 and E-cadherin for proper insulin secretion of MIN6 cells. *Exp Cell Res* 2004;294(2):379-391.
- 44 Calabrese A, Caton D, Meda P: Differentiating the effects of Cx36 and E-cadherin for proper insulin secretion of MIN6 cells. *Exp Cell Res* 2004;294(2):379-391.
- 45 Birchmeier W, Behrens J: Cadherin expression in carcinomas: role in the formation of cell junctions and the prevention of invasiveness. *Biochim Biophys Acta* 1994;1198(1):11-26.
- 46 Perl AK, Wilgenbus P, Dahl U, Semb H, Christofori G: A causal role for E-cadherin in the transition from adenoma to carcinoma. *Nature* 1998;392(6672):190-193.
- 47 Takeichi M: Cadherins in cancer: implications for invasion and metastasis. *Curr Opin Cell Biol* 1993; 5(5):806-811.
- 48 Bertolino P, Tong WM, Herrera PL, Casse H, Zhang CX, Wang ZQ: Pancreatic beta-cell-specific ablation of the multiple endocrine neoplasia type 1 (MEN1) gene causes full penetrance of insulinoma development in mice. *Cancer Res* 2003;63(16):4836-4841.
- 49 Fleischer N, Chen C, Surana M, Leiser M, Rossetti L, Pralong W, Efrat S: Functional analysis of a conditionally transformed pancreatic beta-cell line. *Diabetes* 1998;47(9):1419-1425.
- 50 Luther MJ, Davies E, Muller D, Harrison M, Bone AJ, Persaud SJ, Jones PM: Cell-to-cell contact influences proliferative marker expression and apoptosis in MIN6 cells grown in islet-like structures. *Am J Physiol Endocrinol Metab* 2005;288(3):E502-E509.
- 51 Luther MJ, Davies E, Muller D, Harrison M, Bone AJ, Persaud SJ, Jones PM: Cell-to-cell contact influences proliferative marker expression and apoptosis in MIN6 cells grown in islet-like structures. *Am J Physiol Endocrinol Metab* 2005;288(3):E502-E509.
- 52 Muller D, Jones PM, Persaud SJ: Autocrine anti-apoptotic and proliferative effects of insulin in pancreatic beta-cells. *FEBS Lett* 2006;580(30):6977-6980.
- 53 Yoshitomi H, Fujii Y, Miyazaki M, Nakajima N, Inagaki N, Seino S: Involvement of MAP kinase and c-fos signaling in the inhibition of cell growth by somatostatin. *Am J Physiol* 1997;272(5 Pt 1):E769-E774.
- 54 Luther MJ, Davies E, Muller D, Harrison M, Bone AJ, Persaud SJ, Jones PM: Cell-to-cell contact influences proliferative marker expression and apoptosis in MIN6 cells grown in islet-like structures. *Am J Physiol Endocrinol Metab* 2005;288(3):E502-E509.
- 55 Cox RT, Kirkpatrick C, Peifer M: Armadillo is required for adherens junction assembly, cell polarity, and morphogenesis during *Drosophila* embryogenesis. *J Cell Biol* 1996;134(1):133-148.
- 56 Huelsken J, Vogel R, Brinkmann V, Erdmann B, Birchmeier C, Birchmeier W: Requirement for beta-catenin in anterior-posterior axis formation in mice. *J Cell Biol* 2000;148(3):567-578.
- 57 He TC, Sparks AB, Rago C, Hermeking H, Zavel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW: Identification of c-MYC as a target of the APC pathway. *Science* 1998;281(5382):1509-1512.
- 58 Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R, Ben-Ze'ev A: The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. *Proc Natl Acad Sci U S A* 1999;96(10):5522-5527.
- 59 Tetsu O, McCormick F: Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* 1999;398(6726):422-426.
- 60 Hinck L, Nathke IS, Papkoff J, Nelson WJ: Dynamics of cadherin/catenin complex formation: novel protein interactions and pathways of complex assembly. *J Cell Biol* 1994;125(6):1327-1340.
- 61 Murant SJ, Handley J, Stower M, Reid N, Cussenot O, Maitland NJ: Co-ordinated changes in expression of cell adhesion molecules in prostate cancer. *Eur J Cancer* 1997;33(2):263-271.
- 62 Umbas R, Isaacs WB, Bringuier PP, Xue Y, Debruyne FM, Schalken JA: Relation between aberrant alpha-catenin expression and loss of E-cadherin function in prostate cancer. *Int J Cancer* 1997;74(4):374-377.
- 63 Watabe M, Nagafuchi A, Tsukita S, Takeichi M: Induction of polarized cell-cell association and retardation of growth by activation of the E-cadherin-catenin adhesion system in a dispersed carcinoma line. *J Cell Biol* 1994;127(1):247-256.
- 64 Vasioukhin V, Bauer C, Degenstein L, Wise B, Fuchs E: Hyperproliferation and defects in epithelial polarity upon conditional ablation of alpha-catenin in skin. *Cell* 2001;104(4):605-617.
- 65 Matsubara S, Ozawa M: Expression of alpha-catenin in alpha-catenin-deficient cells increases resistance to sphingosine-induced apoptosis. *J Cell Biol* 2001;154(3):573-584.
- 66 Hwang SG, Yu SS, Ryu JH, Jeon HB, Yoo YJ, Eom SH, Chun JS: Regulation of beta-catenin signaling and maintenance of chondrocyte differentiation by ubiquitin-independent proteasomal degradation of alpha-catenin. *J Biol Chem* 2005;280(13):12758-12765.
- 67 Bouwens L, Rooman I: Regulation of pancreatic beta-cell mass. *Physiol Rev* 2005;85(4):1255-1270.