Generation and characterization of human insulin-releasing cell lines

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Abstract

Background: The in vitro culture of insulinomas provides an attractive tool to study cell proliferation and insulin synthesis and secretion. However, only a few human beta cell lines have been described, with long-term passage resulting in loss of insulin secretion. Therefore, we set out to establish and characterize human insulin-releasing cell lines.

Results: We generated ex-vivo primary cultures from two independent human insulinomas and from a human nesidioblastosis, all of which were cultured up to passage number 20. All cell lines secreted human insulin and C-peptide. These cell lines expressed neuroendocrine and islets markers, confirming the expression profile found in the biopsies. Although all beta cell lineages survived an anchorage independent culture, none of them were able to invade an extracellular matrix substrate.

Conclusion: We have established three human insulin-releasing cell lines which maintain antigenic characteristics and insulin secretion profiles of the original tumors. These cell lines represent valuable tools for the study of molecular events underlying beta cell function and dysfunction.

Background

A major obstacle in beta cell research has been the lack of a human pancreatic beta cell line that is functionally equivalent to primary normal or neoplastic beta cells because of difficulties in obtaining and culturing them for long periods of time [1,2]. Therefore, animal insulinoma cell lines are widely used to study both physiological and pathophysiological mechanisms involved in glucose metabolism and to establish in vitro models for the beta cell damage occurring in type 1 diabetes [3-8]. Nevertheless, recent studies comparing sequences that lie upstream of, or flank the transcription start site of the insulin gene among different species, led to the conclusion that the rodent promoters are markedly different from the human
one, urging caution in extrapolating data from rodent promoter studies to the etiology and therapy of diabetes [9].

Insulinomas are the most common pancreatic islet cell tumors, arising from the beta cells within the islets of Langerhans. Most of them are sporadic [10] and small, displaying a benign behaviour, but still cause substantial morbidity since they produce excessive amounts of hormones. In fact, their key hallmark is uncontrolled insulin secretion, despite hypoglycemia. Compared with normal beta cells, insulinomas also tend to secrete more proinsulin, leading to an increased ratio of proinsulin to insulin [10-12].

Although the clinical association between hypoglycemia and pancreatic beta cell tumors was already described in 1927 [13], the molecular mechanisms involved in this disease remain unknown. Histochemical studies in human insulinomas showed that tumors have reduced insulin protein content compared with normal beta cells; hence it is possible that decreased storage capacity and uncontrolled hormone release are responsible for the hyperinsulinemia [11].

Persistent hyperinsulinemic hypoglycemia of infancy (PHHI), or nesidioblastosis, is a rare disorder characterized by unregulated insulin secretion and profound hypoglycemia [14]. PHHI could also be characterized by the histological appearance of endocrine cells lying in the duct epithelium, with an apparent failure to aggregate into discrete islets of Langerhans [15]. However, there is some controversy on the etiology of the disease because severe hypoglycemia can also occur in the presence of apparently normal islets [16].

To the best of our knowledge, the number of human cells growing in vitro which persistently release insulin is limited [17-19].

In view of the great demand for easily accessible beta cell lines for physiological and medical relevant studies [9], we set out to generate and characterize human insulin-releasing long-term cell cultures derived from biopsies of insulinomas and nesidioblastosis.

Here, we describe three new insulin-releasing low passage cell lines, derived from two independent insulinomas (APM and CPR cell lines) and one nesidioblastosis (VGA cell line) that maintain the antigenic characteristics and insulin secretion profile of the original tissues at least for up to 20 cell passages.

Although the behaviour of these cell lines does not perfectly mimic the primary beta cell physiology, they are extremely valuable tools for developing bioengineered beta cells offering unique opportunities to investigate complex aspects of tumor biology, insulin secretion and beta cell function.

Results

Primary cultures and establishment of cell lines

Three ex-vivo primary cultures (APM, VGA and CPR) were obtained from independent donors after surgical resection of the tissue and cell processing. Cells were successfully grown as monolayers up to passage number 20 (25 weeks approximately), showing fibroblastic-like morphology. All cell cultures were characterized by an overall 2-dimensional growth pattern. Nevertheless, all of them also presented the capacity to form cell clusters (Fig. 1A).

In order to confirm their human origin, diploid condition and chromosomal structure, we karyotyped all three cell lines. Cells at passages 12, 7 and 11 for, respectively, APM, CPR and VGA lines were karyotyped following standard techniques, as described in Material and Methods section. This procedure confirmed the human origin of all three cell cultures and also showed an unaltered number of chromosomes, when compared to normal human cells. Interestingly, CPR cells presented a chromosomal abnormality in mosaicism on chromosome 22 (karyotype of CPR cells: 46, XY, der (22)/46, XY) (Fig. 1B).

Functional analysis and cell lines characterization

In order to approach the cell cultures characterization, we studied, by confocal immunofluorescence (IF) microscopy, several pancreatic islet markers, such as insulin, glucagon, C-peptide, Glut-2 and PDX-1, as well as ductal (cytokeratin 19), mesenchymal (alpha-actin, desmin and vimentin) and one of the endocrine pancreatic tumor (chromogranin) cell markers. As shown in Fig. 1C, while APM cells were positive for chromogranin, PDX-1 and insulin, and VGA cells showed a positive staining for chromogranin, PDX-1, Glut-2 and glucagon; only CPR cells presented a positive staining for chromogranin plus all the beta cell markers analysed (PDX-1, insulin, C-peptide and Glut-2). It is important to note that, almost all of the cells of each cell culture analyzed, presented the same staining pattern described above. Nevertheless, a cell proportion of less than 1% was desmin, alpha-actin, vimentin or CK19-positive (see Additional file 1).

Next, we also investigated the mRNA expression levels of some neuroendocrine and exocrine markers by means of RT-qPCR. In general, all of the genes studied presented positive expression. In order to follow the evolution of the cell cultures, the data shown in figure 2 represent the means of, at least, three experiments performed with mRNA samples obtained from different cell passages. Interestingly, all cell lines presented less than 1% of chromogranin and insulin expression when compared with human islet cultures, set as 100%. As previously reported [17,20], our cell lines also showed lower levels of
Figure 1

**Human beta cell cultures growing in vitro.** Morphological and immunofluorescence studies performed on cells from passages 5 to 15. (A) Cells (1 × 10^4 cells/well) obtained from human insulin secreting cell cultures, were incubated for 4 days in CMRL medium containing 0.5% FCS. 2-dimensional, expansive growth pattern (top panel) and a cluster-like, 3-dimensional type of growth (bottom panel). Representative fields (x100) were photographed under a phase-contrast Nikon microscope. (B) Representative G-banded karyotype of VGA (46, XX) (upper left panel); APM (46, XY) (upper right panel) and CPR cells (46, XY) (bottom left panel) and (46, XY, der(22)) (bottom right panel, black arrow indicates chromosome 22 alteration). (C) Immunofluorescence confocal microscopy. After fixation and permeabilization, the cells were labeled with antibodies against chromogranin A(green) and alpha-actin (red) (top left panels), insulin (green) and desmin (red) (middle left panels), glucagon (green) and desmin (red) (bottom left panels), Glut2 (green) and vimentin (red) (top right panels), C-peptide (green) and vimentin (red) (middle right panels) or PDX1 (green) and cytokeratin 19 (red) (bottom right panels). In all cases nuclei were stained with DAPI (blue). A representative field is shown. Bar: 50 μm.
the beta cell potassium – ATP channels subunit SUR1 (APM: 0.18 ± 0.03%; VGA: 0.16 ± 0.03%; CPR: 12 ± 1%) and reduced levels of the prohormone convertase 1 and 2 (PCSK1 and 2) (for PCSK1: APM: 2.1 ± 0.4%; VGA: 1.36 ± 0.1%; CPR: 58 ± 3%. For PCSK2: APM: 0.3 ± 0.1%; VGA: 0.2 ± 0.1%; CPR: 1.0 ± 0). In agreement with the immunohistochemistry data from the biopsies and the immunofluorescence performed in the cell cultures, glucagon mRNA levels were barely detected only in CPR and VGA cells (Fig. 2). Furthermore, in order to control for the proportion of exocrine pancreatic cells, the cell lines amylase levels were also monitored. All cell lines presented a very low abundance of exocrine cells as shown by the almost undetectable amylase mRNA levels detected (Fig. 2).

Cells lines were periodically monitored, from the beginning of the primary cultures and every three cell passages, for intracellular hormone content and release and, also, for glucose-induced insulin secretion to select for functional beta cell populations. As shown in Table 1, all three cell cultures presented greatly increased accumulated insulin and proinsulin release (approximately 100 times the values obtained from the primary cultures of human pancreatic islets). Moreover, in accordance with the described behaviour of insulinomas, decreased C-peptide release and barely detectable insulin content were also observed in these cultures. Furthermore, no insulin secretion response was observed upon exposure of the cells to high concentrations of glucose (20 mM), showing no capacity to respond in a physiological manner to increased glucose concentration (Table 1).

**Cell growth properties**

In order to establish the growth parameters of the cell cultures, we compared their growth in the presence of 10% FCS and determined the doubling times for each one of them. As shown in figure 3, APM, CPR and VGA cells displayed similar growth curves, presenting a doubling time of approximately 40 h (APM: 38.5 ± 0.3 h; VGA: 40.5 ± 0.3 h; CPR: 42 ± 1 h).

We also set out to study the cell behaviour of these cultures upon treatment with a panel of growth factors and hormones, which have been described as proliferation inducers in cancer. We determined that all three human cell lines cultures presented similar proliferation patterns. While none of them responded to epidermal growth factor (EGF) at any tested concentration; either Insulin-like growth factor 1 (IGF-1) or recombinant human prolactin (rhPRL) induced a significant growth increase (p < 0.05) on APM, CPR and VGA cells, the highest stimulation rate being exerted at 20 ng/mL and 300 μg/mL for IGF-1 and rhPRL, respectively (Fig. 3B–D). Moreover, simultaneous stimulation with rhPRL and IGF-1, at suboptimal concentrations, had no additive effect on cell proliferation (Fig. 3E).

Next, we evaluated the clonogenic ability of the cell lines, defined as the capacity of isolated cells to form colonies. We demonstrated that VGA, CPR and APM cells were unable to form colonies when seeded at low density (data not shown).

On the other hand, the anchorage-independence was also tested by seeding the cells onto an agar overlay. As shown in figure 4, when adhesion to a substrate was prevented, all cell lines formed spheroids, but only APM cells were able to proliferate under this condition (evaluated at 4 days after seeding).

VGA, CPR and APM cells growing in 96-well plates were incubated with increasing doses of Doxorubicin (Doxo) (0.75, 1.5, 3, 6 and 12 μM), and cell viability was evaluated 48 h after treatment by means of MTS, as described in Material and Methods. We observed that only CPR cells were sensitive to Doxo, exhibiting an IC50 of 5.1 μM (Fig. 5A).

The susceptibility of the human beta cell lines to serum starvation was also studied. Subconfluent monolayers were incubated for 96 h in the absence of FCS and the viability was then measured using MTS assay. As shown in figure 5B, APM cells were 1.5 fold more susceptible to serum starving than VGA cells.

**Adhesiveness ability and expression of adhesion molecules**

We first examined the adhesiveness ability of CPR and APM cells to different substrates. As shown in figure 6A adhesion to plastic, to control substrate, to fibronectin (FN) and to Col IV was similar between all of the human beta cell lines. More over both insulinoma cell cultures presented more adhesiveness to Laminin (LN) (Fig. 6A).

Integrins are a family of cellular transmembrane receptors that recognize different extracellular matrix components, such as FN. By Western blot (WB) and IF analysis, we show that all insulinoma cell lines express similar levels of β1-Integrin (Fig. 6B).

Since the expression of E-Cadherin, a 120 kDa transmembrane glycoprotein, is associated with cell-cell adhesion, we next examined its expression at the protein level. We observed that only APM cells were positive for E-Cadherin, as detected by WB and IF (Fig 6B). Classical cadherins are defined by their ability to form complexes with catenins. We found that all insulinoma cell lines expressed β-Catenin, without significant differences among them (Fig. 6B).
Figure 2

mRNA expression levels of endocrine and exocrine markers. Insulin, chromogranin, SUR1, PCSK1, PCSK2, glucagon, and amylase were measured by Real-time RT-PCR reaction using SYBR® Green Dye reagent (Applied Biosystems, Foster City, CA, USA). All reactions were performed in triplicates ($n = 4$ independent experiments). The relative expression of a target gene was determined in comparison to a reference gene (HPRT). In addition H-MBS mRNA-expression level was used as a positive control for similar expression levels between cell lines and human islets. Results are presented as relative increase where the value obtained from primary cultures of human islets was set to 100. $a p < 0.001$. APM, VGA or CPR cells vs. human islets. For SUR1 and PCSK1 mRNA levels: $a p < 0.001$. APM, VGA or CPR cells vs. human islets, and also CPR vs. APM or VGA cells. ANOVA/Bonferroni’s tests.

Table 1: Accumulated insulin, C-peptide and pro-insulin release and insulin content.

|                      | VGA       | APM       | CPR       | hBeta-cells |
|----------------------|-----------|-----------|-----------|-------------|
| Insulin release (ng/ngDNA) | $5 \times 10^{-2} \pm 1 \times 10^{-2}$ | $2.5 \times 10^{-2} \pm 3 \times 10^{-3}$ | $9 \times 10^{-3} \pm 1 \times 10^{-3}$ | $9 \times 10^{-3} \pm 1 \times 10^{-3}$ |
| Insulin content (ng/ng DNA) | $<3 \times 10^{-7}$ | $<3 \times 10^{-7}$ | $<3 \times 10^{-7}$ | $3 \times 10^{-4} \pm 1 \times 10^{-4}$ |
| Pro-Insulin release (pmol/ng DNA) | $8 \times 10^{+4} \pm 2 \times 10^{-4}$ | $3 \times 10^{+4} \pm 3 \times 10^{-5}$ | $3 \times 10^{+4} \pm 1 \times 10^{-5}$ | N.D. |
| C-peptide release (ng/ng DNA) | $5 \times 10^{+2} \pm 1 \times 10^{-2}$ | $5 \times 10^{+2} \pm 1 \times 10^{-2}$ | $5 \times 10^{+2} \pm 1 \times 10^{-2}$ | $5 \times 10^{+2} \pm 1 \times 10^{-2}$ |
| Glucose-induced insulin secretion (Stimulation Index)$^*$ | [I] | [I] | [I] | $>3$ |

$^*$ Stimulation Index = Insulin secretion (20 mM glucose): Insulin release (2.8 mM glucose)

N.D.: not detected
Spreading, migration and invasion abilities

The percentage of spreading cells was determined 45 min after plating by scoring cell morphology under a phase-contrast microscope. We determined that while 60% of VGA cells showed a flattened round base and lamella or filopodia extensions (spreading group), APM and CPR cells presented only 30% of spreaded cells (Fig. 7A).

"Wound-healing" assay was used to analyze the in vitro migration ability of the human beta cell lines. Four hundred μm wide wounds were made in confluent monolayers of the different cell cultures, including primary cultures of human normal islets, and migration into the cell-free area was observed and measured. While at 18 h after the initial wound was made VGA cells had covered about 30% of the cell-free area, CPR and APM cells showed a significantly lower migratory ability, covering less than 15% of the original wound in the same time period. Interestingly, we determined that primary cultures from normal human islets completely closed the wound during this same time period (Fig. 7B).

We also analyzed the invasive capacity using "Transwell" chambers, with filters of 8 μm pores covered with the reconstituted basal membrane Matrigel. Twenty two hours after seeding, the number of insulinoma cells that invaded the
Matrigel and the filters was evaluated by means of an inverted microscope. However, no invasion was found either in AMP, VGA or CPR cells (data not shown).

Proteases secretion profile
Proteases are expressed in normal processes but also, in a non-regulated manner, during pathological events as tissue invasion and metastasis. Among the principal proteases associated with the invasive capacity of tumor cells are the metalloproteinases (MMPs) and the serine protease uPA. Therefore, we evaluated uPA, MMP-9 and MMP-2 activity secreted to the conditioned medium of the human cell lines. All cell lines were able to secret MMP-9, with no differences among the cell types (Fig. 8). However, none of the analyzed cells appeared to be secreting MMP-2 (data not shown).

Constitutive uPA activity was very low in all cell types, being detectable only in VGA cells (3.08 × 10^{-5} ± 6.9 × 10^{-6} UI/μg of protein, data not shown).

Discussion
Most insulinomas are derived from monoclonal proliferation of pancreatic islets beta cells; therefore, they constitute good models for studying both tumor and beta cell biology.

Here, we report the morphological and cytogenetic features, as well as the biological behaviour, of three long term cultures of insulin producing human cells derived from two isolated insulinomas and one nesidioblastosis case. To the best of our knowledge, the number of in vitro grown, persistently insulin-secreting human cells is very limited. Recently, the
human glucose-responsive insulin secreting NES2Y cell line, derived from a patient with persistent hyperinsulinemic hypoglycemia of infancy, was established. This cell line was characterized as presenting a population doubling time of approximately 24 h, an insulin promoter unresponsive to glucose, a lack of ATP-sensitive potassium channel and a defect in the PDX1 insulin gene-regulatory transcription factor [17,20,21]. Moreover, two years ago, Gartner and collaborators described a few characteristics of eight long-term insulin-secreting cell cultures maintained in vitro [19]. So far, the only human insulinoma-derived cell line (CM) lacks persistent and glucose-induced insulin secretion and presents several chromosomal abnormalities, specifically including the chromosome 11 tetraploidy, with three out of four chromosomes 11 being aberrant and showing a chromosomal material translocation at a specific locus of the human insulin gene [2,22].

The cell lines described here displayed the same antigenic and insulin secreting profiles as the original tumors. It is important to note the fact that the barely detectable intracellular insulin levels is in accordance with the description of insulinomas as having poor or almost non-existent capacity for insulin storage, thus presenting no positive staining for insulin [20,23]. Unlike the CM cells, our cell cultures presented large amounts of human insulin secretion which was maintained throughout the different passages, in addition to an unaltered number of chromosomes and only an insertion in mosaicism on chromosome 22 in the CPR cells.

In agreement with the very well characterized excessive and/or uncontrolled insulin release, displayed by insulinomas and nesidioblastosis [24,25], none of our cell cultures presented glucose-induced insulin secretion. It has been suggested that the histological resemblance of nesidioblastic pancreata to those of immature fetuses may have arisen as the result of inappropriate control during the earliest phases of endocrine pancreatic development [26]. Further results supported this conclusion, adding that in nesidioblastosis, this similarity is not only anatomic, but also functional, because of their defective recognition of nutrient secretagogues, and lack of glucose dependence of their cAMP mediated insulin release [27]. Furthermore, the lack of glucose-regulated insulin secretion, observed in our human insulin-producing cell lines, might be attributed to the loss of the sulphonylurea receptor gene (SUR1) which would lead to the absence of functional ATP-sensitive potassium channels.

The elevated levels of proinsulin detected in our cultures, could be related on one hand, to diminished mRNA levels of prohormone convertase 1 and 2 displayed in these cell lines. These enzymes are responsible for cleavage of proinsulin and thus are involved in the presence of this insulin precursor in secretory granules. On the other hand, it has already been reported that unlike normal pancreatic beta cells, in which proinsulin to insulin conversion occurs in acidic immature secretory granules of the trans-Golgi apparatus, in insulinomas, this conversion already takes place in the Golgi apparatus, but remains incomplete, resulting in the formation of secretory granules containing both proinsulin and insulin. This indicates that in insulinomas, sorting into secretory granules may not be a prerequisite for hormone conversion [20].

It is important to note that, probably due to their benign nature, the cell lines generated in our work presented sev-
eral growth features, including dependence of trophic factors, reduced clonogenic ability and capacity to form cell clusters, which are similar to those found in human normal islet primary cultures. The human insulin-releasing cell lines described here presented a relatively low proliferation rate, which appears to be in agreement with the already published data and also with the rate found in human islet primary cultures [20,28]. Moreover, this low proliferation rate could explain the resistance to doxorubicin treatment observed in our cultures, since this kind of compounds has been associated with low success rates in well-differentiated tumors [29] and are only used in malignant insulinoma treatment [30].

Among the panel of growth factors tested, only IGF-1 and rhPRL were able to induce cell proliferation of our cell lines. Several studies have pointed IGF-1 as a growth promoter for beta cells [31-33]. Furthermore, the mitogenic activity of the lactogen family of hormones, which include prolactin, have also been described for normal and neoplastic human beta cells [19,28].

Metastasis is the process by which tumor cells establish new tumor foci at different and distant organs. This is a very complex multistep process, in which each step is crucial and strictly regulated. Acquisition of invasive ability involves changes in adhesion, migration and extracellular

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**Figure 6**

*Adhesiveness ability and pattern of expression of adhesion molecules.* (A) APM and CPR cells were allowed to attach to FN, LN, Col IV or BSA (control) coated wells. The rate of cell adhesion at 2 h was expressed as the percentage of the total number of cells seeded. The results are representative of three independent experiments. Each value represents the mean ± SD of triplicates. a p < 0.001 APM FN vs APM plastic, and CPR FN vs CPR LN, CPR plastic and CPR BSA. b p < 0.005 APM FN vs APM BSA, and APM LN vs APM plastic. c p < 0.05 APM FN vs APM Col IV, APM LN vs APM BSA, APM LN vs CPR LN, and CPR FN vs CPR Col IV. ANOVA/Bonferroni’s tests. (B) Expression of β1-Integrin, E-Cadherin and β-Catenin was analyzed by Western blot and Immunofluorescence as described in Materials and Methods. Representative images are shown.
proteolysis, culminating in shedding of tumor cells into the circulation [34]. Few studies focusing on cell migration and invasion properties of endocrine pancreatic tumor cells (EPTs) are available. We evaluated every step of this process in our cell models.

Firstly, the migration capacity of our cell cultures was compared with that displayed by human islet primary cultures. The results showed that even when all the insulin-releasing cultures presented very low migration, the motility of the neoplastic cells was very low, and surprisingly, this ability increased in hyperplastic (nesidioblastosis)-derived VGA cells. This result is in agreement with the higher spreading capacity detected in these cells. The highest migration values were obtained in primary cultures of normal human islets. It remains to be elucidated which are the causes of this unexpected cell behaviour.

We next examined the adhesiveness ability of CPR and APM cells, determining that adhesion to plastic, to control substrate, to FN and to Col IV was similar between all of the human beta cell lines. Both insulinoma cell cultures were more adhesive to LN. The differences found in the adhesion pattern could be explained by variations in the expression profiles of adhesion molecules. FN is an extracellular protein which binds cells via specific integrins. In agreement with the similar adhesiveness to FN, we found no differences in β1-Integrin expression between the cell lines. Moreover, the only report we found, focused on down regulation of E-cadherin, a homotypic cell-to-cell interaction molecule ubiquitously expressed in epithelial cells, reported on the RIP-Tag model but, so far this has not been confirmed by studies on primary EPTs [35]. In our work, we found a lack of E-Cadherin expression in two out of three human beta-cell lines studied. We showed, for the first time, that the APM human insulinoma derived cells, the only one presenting positive staining for E-cadherin, also displayed the lowest MMP-9 activity. These facts could, at least in part, explain the poor migration ability of this cell line, correlating with the benign behavior of the original tumor. Cadherins are linked to the actin cytoskeleton through catenins and, in particular, β-Catenin binds directly to the intracellular domain of E-Cadherin. Surprisingly, and in spite of the differences found in the E-cadherin expression, all insulinoma cells presented similar levels of β-Catenin. Therefore, it is possible that the β-Catenin found in the VGA and CPR cells is involved in other cellular functions, independently of adhesion.

**Figure 7**

Cell spreading and migratory ability. (A) Cells from the different human insulin-producing cell lines were seeded and allowed to adhere for 60 min. After removal of non adherent cells, the percentage of spread-out cells was determined at 45 min. Data were collected by random observation and at least in triplicate. a p < 0.001. ANOVA/Bonferroni’s test. (B) Confluent monolayers of the human cell lines and of normal islets were "wounded”. The cells were allowed to migrate into the cell-free area for 18 h and then photographed. Cell migration was evaluated by densitometry, calculating the area occupied by the migratory cells. Migration is expressed as the percentage (mean ± SD) of wound covered area. a p < 0.001 normal islets vs cell lines. b p < 0.001 VGA vs APM and CPR cells. ANOVA/Bonferroni’s test.
Overall, the results related to cell invasion and migration of these cultures are in agreement with the ones found in hyperplasic cells or benign tumors from which these cultures were derived, indicating that the cells maintained the original characteristics throughout the cell passages.

These human insulin-releasing cell lines have been characterized in detail, and express several key features of normal pancreatic beta cells, such as expression of a number of islet genes (e.g.: insulin, PDX-1, Glut2, Prohormone convertases, glucagon) and formation of cell clusters.

**Conclusion**

The cell lines described in this work represent, even when their behaviour do not perfectly mimic the primary beta cell physiology, valuable tools for the study of the molecular events underlying beta cell function and dysfunction. The merits which become evident from the characterization and use of APM, CPR or VGA cells in diverse functional studies reported here, should be balanced against the short lifespan of primary beta cells which also undergo a rapid decline in their function and granulation with time in culture.

It is important to say that, we are currently performing comparative proteomic studies, in order to further characterize the differences between normal human islets cultures and these cell lines.

Collectively, these observations prompt research towards successful establishment of bioengineered human beta cells, providing a large and much needed source of cultured human beta cell tissue for experimentation. More over, the cell lines described here could also be useful to improve the knowledge on the molecular biology of insulinomas in order to select and/or validate new therapeutic targets and diagnostic biomarkers.

**Methods**

**Patients’ data**

Three ex-vivo primary cultures (APM, CPR and VGA cells) were obtained from independent donors after surgical resection and tissue processing. The tissue samples were processed after informed consent was obtained from patients enrolled in a protocol of the bile ducts and pancreas service, approved by the institutional board of the Hospital das Clínicas, FMUSP. APM and CPR cultures were established from biopsies of isolated insulinomas of men aged 27 and 22 years old, respectively. The diagnosis of insulinoma was confirmed by high insulin, C-peptide, and proinsulin plasma concentrations with low glucose levels during a 19 h supervised fast. The tumors were localized by calcium-stimulated selective arteriography. Both patients underwent a curative surgical tumor resection that resulted in full resolution of their symptoms. The tumor diagnosis was further confirmed by immunohisto- logical analysis. All tumors stained for insulin, chromogranin and synaptophysin.

VGA cells were obtained from a woman aged 23 years old presenting no tumor and high insulin, C-peptide, and proinsulin plasma concentration with low glucose levels during a 19 h supervised fast. A partial pancreatectomy was performed in this patient and nesidioblastosis was diagnosed by means of histopathological analysis. The biopsy presented positive staining for insulin, glucagon, somatostatin, chromogranin and synaptophysin.

**Primary cultures derived from insulinomas and nesidioblastosis**

Primary cultures were obtained by mechanical fragmentation and enzymatic digestion of solid fragments with Liberase (Roche biodiagnostics, Indianapolis, IN). Several fractions containing different cell populations were separated by serial sedimentation and centrifugation steps. The cells were plated in culture flasks with CMRL 1066 (Mediatech-Cellgro, Miami, FL) supplemented with ITS (insulin 10 mg/L, transferrin 5.5 mg/L, sodium selenite 0.0067 mg/L, ethanolamine 2 mg/L, Invitrogen, Carsbad, CA), penicillin 100 units/mL, 10% fetal calf serum (FCS, Cultilab, Campinas, SP, Brazil) and glucose (final concentration 11 mM) and allowed to attach for 24 to 48 h. In all cases, culture media were changed every 2–3 days.
At confluence or when every cell clusters appeared overcrowded, the cells were detached with 0.025% trypsin, washed, and resuspended in fresh medium. All cell culture experiments were performed at 37 °C in a humidified atmosphere containing 5% CO2.

Human islet isolation and cell culture: Human pancreas from adult brain-dead donors (mean age 50 ± 3 years, n = 7) were harvested in conformity with Brazilian regulations and the local Institutional Ethical Committee. Pancreatic islets were isolated by the automated method of Dr. Ricordi and collaborators [36] with previously described modifications [28,37].

The islets were first maintained in CMRL 1066 medium supplemented with 100 units/ml penicillin and 5% fetal calf serum FCS at 37 °C in a humidified atmosphere containing 5% CO2.

**Immunofluorescence (IF)**

Cultured cells were trypsinized and plated onto glass coverslips in a 24-well plate at a density of 1 × 10^4 cells/well and grown for 3–7 days. Cells were then washed, fixed, permeabilized and incubated with the corresponding antibodies as previously described [28]. The following primary antibodies were used: rabbit polyclonal anti-hInsulin (ICN, Aurora, OH), rabbit polyclonal anti-homeobox domain transcription factor Pdx1 [36] (kindly donated by Dr. C.V. Wright, Department of Cell Biology, School of Medicine, Vanderbilt University), rabbit polyclonal anti-hGlucagon (Zymed Laboratories Inc., San Francisco, CA), mouse monoclonal anti-hCytokeratin 19 (ICN, Cosa Mesa, CA), mouse monoclonal anti-Vimentin (Sigma, St. Louis, Mo), mouse monoclonal anti-Desmin (Sigma, St. Louis, Mo), mouse monoclonal anti-alpha Actin (Sigma, St. Louis, Mo), rabbit polyclonal anti-hGlut2 (Chemicon, Temcula, CA), rabbit polyclonal anti-hChromogranin A (DakoCytomation, Danemark), rabbit polyclonal anti-h-C-peptide (Linco Research, St. Charles, MO), mouse monoclonal anti-α1-Integrin (clone W1B10, Sigma), mouse monoclonal anti-E-Cadherin (clone 32, Transduction Laboratories) and mouse monoclonal anti-β-Catenin (clone 14, Transduction Laboratories). The primary antibodies were detected with fluorescent labeled secondary antibodies as previously described [28]. The following primary antibodies were detected with fluorescent labeled secondary antibodies (Vector Laboratories, Burlingame, CA) and DAPI or Propidium Iodide (PI) was used for nuclear staining. The immunostained coverslips were examined using a confocal laser scanning microscope LSM 510 (Carl Zeiss GmbH, Jena, Germany). Images selected were representative of the majority of the cells present on each experiment (n = 3 experiments performed).

Control experiments to test primary antibodies specificity included incubation with pre-immune mouse and rabbit sera. All confocal immunofluorescence microscopy images presented correspond to single optical sections.

**RTqPCR**

Cells were harvested and total RNA were prepared and checked for integrity as previously published [39]. cDNA was generated from the RNA samples using SuperScript (Invitrogen, Carlsbad, California). The primers used for gene amplification by qPCR experiments were designed using the Primer Express 3.0 Software (Applied Biosystems, FosterCity, California) connected to the 7300 real time PCR-system Thermocycler (Applied Biosystems, Foster City, CA). The following primers were used in this study: hInsulin (F:5’TCCGGGGAACGAGGCTTCTCTA3’; R:5’AGGGACCCCTCCAGGGCGGAAG3’), hGlucagon (F:5’ATATCTT GCCCGAGGGACIT3’; R:5’ACGTTGCCTAGGTTGTA TGITT3’), hAmylase (F: 5’CTCGGCACAGTTAICTGCGAAGT 3’; R:5’CGCTCT TGTCGATAC GAAAC3’), hPro-hormone convertase 2 (F:5’CCAACAT TAATGTCCGAAGCAAGT3’; R: 5’CCGTGGCTTTAAC-CAGT CA3’), hPro-hormone convertase 1 (F: 5’ATATTCCGCGAAG AGGAGACCITCA3’; R: 5’GCAATAGGAGAT GTATCCGTTCT3’), hChromogranin A (F:5’AAACAGCGGTTTGAAGAAGTA ACT3’; R: 5’CTCCFATAC AACITCGTGGATGAGTAC GGCITG GTCT3’), hSUR1 (F: 5’TGGACCTG GTTGTTCACTCCTT CATGGATCT3’; R: 5’CTTACCTCCITCGGTGCTTCTA3’), hMbs (F:5’TGGACCTCTGTGTGTACCCITCCTT; R: 5’CAACGACATCATGAGGGTT TTC) and hHypoxanthine phosphoribosyl transferase (HPRT) (F:5’GAAATCTTG TGTCGATCCITCTAT GGGATGCT3’; R: 5’CTTCATCCITCGGTGCTTCTA3’). To quantify the products formed during the RTqPCR reaction, the SYBR® Green Dye reagent (Applied Biosystems, Foster City, CA, EUA) was used [40]. Assessment of data generated during amplification was performed using the 7300 Real-time PCR System Sequence Detection Software (Applied Biosystems, Foster City, CA). All reactions were performed in triplicates (n = 4 independent experiments). The relative expression of a target gene was determined in comparison to a reference gene [41].

**Accumulated insulin, C-peptide, proinsulin release and insulin content determination**

Cells were cultured in medium supplemented with 100 units/ml penicillin, 0.5% FCS (glucose final concentration: 5.6 mM) for 4 days. Accumulated insulin and C-peptide release were measured by the electrochemiluminescence assay ELECSYS(Roche Diagnostics, Indianapolis, IN)[42]. Accumulated proinsulin release was measured by radioimmunoassay at the São Paulo Radioimmunoassay Centre (CRIESP). Cells were then lyzed to determine insulin and DNA content as described elsewhere [28].

**Glucose-induced insulin secretion**

Cells were cultured in adherence, as previously described. Cultures were washed with PBS and incubated for 2 h with RPMI 1640 without glucose (Cultilab, Campinas, Brazil) supplemented with 0.5% BSA and 2.8 or 20 mM glucose.
Secreted insulin was quantified in media collected from each well by ELECSYS. The stimulation index for each group of cultures was calculated by dividing the value of insulin secretion at 20 mM glucose by that obtained upon 2.8 mM glucose incubation.

Cytogenetics
Semiconfluent cultures were treated with 0.1 μg/mL N-deacetyl-N-Methylcolchicine (Life Technologies Inc., Carlsbad, CA) for 2 h at 37°C and detached with trypsin. Hypotonic treatment was performed in 0.075 M potassium chloride for 10 min at 37°C, and then cells were fixed with 3:1 methanol: glacial acetic acid, dropped on slides and air-dried. The slides were stained with 3% Giemsa (Sigma Chemicals Co.). GTG and CBG banding were performed according to routine techniques [43].

Doubling time
The doubling time of the in vitro cell cultures was determined by plating the 2 × 10³ cells/well in six-well plates. After 24, 48, 72, 120 and 168 h, triplicate cultures from each time-point were fixed and counted. The population doubling time was calculated from the log phase of the growth curve.

Cell proliferation
4 × 10⁴ viable cells/mL were seeded, in triplicate, in 24 multiwell plates in medium supplemented with 10% FCS. After 24 h, the cells were serum starved in CMRL supplemented with 0.1% FCS for another 24 h and then subjected to a 48 h treatment with different growth factors or their corresponding vehicles, as negative controls (EGF or IGF: 0.2 g/mL, 2 ng/mL, 20 ng/mL and 200 ng/mL; rhPRL: 20 μg/mL, 100 μg/mL and 300 μg/mL). Cells were then harvested with trypsin-EDTA, fixed with 3.7% formaldehyde and counted in an electronic CC530 cell counter (CELM, Sao Paulo, SP, Brasil).

Clonogenic assay
0.8 × 10³ and 1.6 × 10³ monodispersed cells were seeded on 6-multiwell plates (Corning, NY) in medium containing 10% FCS. Medium was changed every 72 h. After 10 days of seeding, monolayers were washed, fixed with 5% acetic acid in methanol and stained with crystal violet. Plating efficiency was defined as the percentage of cells which were able to grow as colonies of more than 10 cells.

Anchorage-independent growth
To evaluate cell-cell adhesion, 24-multiwell plates were coated with a 1% agar (Invitrogen life technologies, Carlsbad, CA) underlayer to prevent attachment. 3 × 10⁴ VGA, CPR and APM cells, were seeded in suspension in complete medium containing 10% FCS. At 4 days, the spheroids formed were counted.

Serum depletion
The effect of serum withdrawal was assayed by seeding 1 × 10⁴ cells in a 96-multiwell plate in medium containing 10% FCS and, after 24 h, washing with PBS and cultured cells in serum-free medium for 96 h. Cell viability was assessed by reduction of the tetrazolium salt (MTS) to the formazan product in viable cells (Cell Titer 96 TM, Promega Corp) as calculated by the 492/620 nm absorbance ratio.

Doxorubicin treatment
1 × 10⁴ VGA, CPR and APM cells were seeded in 96-multiwell plates in medium containing 10% FCS. Subconfluent monolayers were washed with PBS and cultured in the presence of 0.75, 1.5, 3, 6 and 12 μM of Doxorubicin for 48 h. The susceptibility to this drug was evaluated by MTS assay. The IC50, that represents the concentration of a drug that is required for 50% reduction in cell viability, was calculated for each cell line.

Adhesion to plastic, BSA, fibronectin (FN), laminin (LN) and Collagen type IV (Col IV)
Wells from 6 multiwell plates were coated with FN (2 μg/cm²) (Sigma), LN (4 μg/cm²) (Sigma) and Collagen type IV (Col IV, 2.5 μg/cm²) (Sigma) in PBS at room temperature for 60 min. Then, 3 × 10⁴ cells per well were seeded in serum free medium and incubated for 2 h at 37°C. Adherent cells were washed with PBS, trypsinized and counted in triplicates. The rate of cell adhesion was expressed as the percentage of the total number of cells seeded.

Analysis of cell adhesion molecules
Adhesion molecules expression was evaluated by IF and Western blot (WB), as described by Peters et al. [44]. Confluent monolayers were washed with PBS and then lysed with Lysis Buffer (PBS-1% Triton X-100) containing protease-inhibitors (10 μg/mL PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 μg/mL pepstatin). WB analyses were carried out using PVDF membranes (BioRad, Hercules, CA). Blocked membranes were then incubated with specific antibodies (anti-β1-Integrin, anti-E-Cadherin, anti-α-Catenin and anti-Actin) and then incubated with peroxidase-conjugated secondary antibody (Amersham-Phar- macia-Biotech). Chemiluminescence was detected using ECL reagent kit (Amersham-Phar-macia-Biotech). Especific bands were analyzed by densitometry (OD) (Molec-ular AnalystTM GS-700, BioRad).

Cell spreading assay
About 5 × 10⁴ cells from all cell lines were seeded onto plastic Petri dishes (35 mm, Corning, NY) and allowed to adhere for 60 min. Non-adherent cells were removed through washing with PBS and the percentage of spread-
ing cells was determined at 45 min by scoring 200–300 cells under a phase-contrast microscope (Nikon, Tokyo, Japan). Data were collected by random observation at least in triplicate. Rounded refringent cells were included in the non-spreading group, and cells with a flattened round base or with lamella or filopodia were included in the spreading group. The rate of cell spreading was expressed as the percentage of the total number of cells seeded.

**Migration assay**

To determine the migratory capacity of the different cell lines, a wound-healing assay was used [44]. Briefly, 5 × 10⁵ cells were seeded onto 60 mm plastic dishes in medium supplemented with 10% FCS. When cells reached confluence, parallel wounds of about 400 μm width were made on the monolayers using a pipet tip. At time 0 and after 18 h of incubation, photographs of the same area were taken to determine the wound-healing level. Images were evaluated by the image-Pro Plus 5.1 software (Media Cybernetics Inc., Bethesda, MD).

**Cell invasion**

The invasive capacity of each cell line was studied using "Transwell" chambers (Corning), in 24 well plates. Filters of 8 μm/pore, were covered in their basal face with 0.1% of gelatin, and on the top face with 250 μg/ml of basal membrane reconstituted Matrigel (Becton Dickinson Labware). FN, (8 μg/ml, Sigma) was used as chemoattractant. 5 × 10⁵ cells were seeded onto the filter, in medium containing 2% FCS. After 22 h at 37°C in a 5% CO₂ atmosphere, cells on the upper surface of the filters were removed by wiping with cotton swabs and filters were removed, fixed with formaldehyde and stained with haematoxylin and eosin. We considered invasive, those cells able to pass through the pores to the other face of the filter.

**Preparation of conditioned medium**

To evaluate secreted proteases, conditioned medium was prepared as previously described [44]. Semi-confluent monolayers in 24 well plates, corresponding to the different cell lines, were extensively washed with PBS to eliminate serum traces. 200 μl of serum-free D-MEM was added and the incubation was continued for 24 h. Conditioned medium was individually harvested from each well and the remaining monolayers were scraped and lysed. Cell protein content was measured by Bradford method.

**Quantification of uPA activity by radial caseinolysis**

A radial caseinolytic method was employed, as previously described [45]. Plasminogen-free casein-agarose gels were used to test plasminogen-independent activity. Briefly, mixtures of 1.25% agarose in deionised water, 20 mg powdered fat-free milk as a casein source, 2 μg/mL plasminogen (Chromogenix) and 100 mM Tris-Cl (pH = 8) were prepared and kept at 46°C. The diameters of the lytic zones were referred to a purified urokinase (Serono) standard curve ranging from 0.2 to 25 IU/mL, and normalized to the original cell culture protein content.

**Detection of MMP activity by quantitative zymography**

Collagenolytic activity secreted by the different insulinoma cell lines was determined in 9% SDS-PAGE copolymerized with 0.1% heat-denatured type I collagen (gelatin, Invitrogen life technologies, Carlsbad, CA BRL), as previously described [46,47]. After running, gels were washed in 2% Triton X-100 and incubated for 72 h at 37°C in 0.25 M Tris - HCl/1 M NaCl/25 mM CaCl₂ buffer (pH 7.4) for specific activity detection, or in the same solution containing 40 mM EDTA to detect non-specific activity. Gels were fixed and stained with Coomassie Blue R-250 and destained in 30% methanol-10% acetic acid in water. Gelatinolytic bands, visualized by negative staining, were densitometered with an image analyzer (Bio-Rad Densitometer, model GS-670). MMP activity is expressed as arbitrary units (AU) per mg cell protein.

**Statistical analysis**

All experiments were performed at least in triplicate. The significance of differences between groups was calculated by applying ANOVA and Bonferroni’s test, as indicated. A value of p < 0.05 was considered to be significant.

**List of Abbreviations**

Upa: urokinase-type plasminogen activator; MMPs: matrix metalloproteinases; CM: conditioned medium; EPT: endocrine pancreatic tumor; IF: immunofluorescence; WB: western blotting; MTS: methyl tetrazolium salt; RTqPCR: Quantitative reverse transcribed real-time polymerase chain reaction.

**Authors’ contributions**

LL: conceived the study, participated in its design, coordination and drafted the manuscript. Also participated in: cell culture, human islets isolation, cell proliferation assays, doubling time calculation, IF: cytogentic studies and hormone determinations. MGP: participated in the design, coordination of the study and drafted the manuscript. She also participated in the characterization of cell growth properties and in the characterization of adhesiveness, spreading, migration and invasion abilities of the cell lines. KK: participated in the obtention of the primary cultures, human islets isolation, doubling time calculation, IF, and RTqPCR. IS: participated in the characterization of cell growth properties, in the determination of adhesiveness, spreading, migration and invasion abilities of the cell lines. He also participated in the protease secre-
tion and the adhesion molecules profiling. LFT: participated in the cell cultures, IF, hormones determinations, cytogenetic studies and RTqPCR. CB: participated in the characterization of cell growth properties and in the characterization of adhesiveness, spreading, migration and invasion abilities of the cell lines. She also participated in the protease secretion and the adhesion molecules profiling. MCM: Participated in the obtention of the biopsies and the interpretation of the data from the patients. LP: participated in the design of the study and in revising the manuscript critically. EBK: Participated in the design and the coordination of the study and helped with the draft of the manuscript. MCS: participated in the design and the coordination of the study and helped with the draft of the manuscript. All authors read and approved the final manuscript.

Additional material

Additional file 1
Positive controls for mesenchymal proteins staining. The data provided represent images form cells presenting positive staining for Desmin, alpha-actin, cytokeratin 19, vimentin in the Confocal Microscopy experiments. Click here for file: [http://www.biomedcentral.com/content/supplementary/1471-2121-10-49-S1.tiff]

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