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Phenotypic and functional characterization of glucagon-positive cells derived from spontaneous differentiation of D3-mouse embryonic stem cells

NESTOR VICENTE-SALAR[1], ALFREDO SANTANA[2], PABLO JUAN-PICÓ[1], JUAN A. REIG[1] & ENRIQUE ROCHE[1]

[1]*Institute of Bioengineering, University Miguel Hernandez, Elche, Spain, and* [2]*Research Unit, Gran Canaria Hospital Dr Negrin and Genetic Unit, Childhood Hospital, Las Palmas, Canary Islands, Spain*

Abstract

Background. Glucagon expression is being considered as a definitive endoderm marker in protocols aiming to obtain insulin-secreting cells from embryonic stem cells. However, it should be considered that *in vivo* glucagon is expressed both in definitive endoderm- and neuroectoderm-derived cells. Therefore, the true nature and function of *in vitro* spontaneously differentiated glucagon-positive cells remains to be established. *Methods.* D3 and R1 mouse embryonic stem cells as well as α-TC1-9 cells were cultured and glucagon expression was determined by real-time PCR and immunocytochemistry. Functional analyses regarding intracellular calcium oscillations were performed to further characterize glucagon$^+$ cells. *Results.* Specifically, 5% of D3 and R1 cells expressed preproglucagon, with a small percentage of these (<1%) expressing glucagon-like peptide 1. The constitutive expression of protein convertase 5 supports the expression of both peptides. Glucagon$^+$ cells co-expressed neurofilament middle and some glucagon-like peptide-1$^+$ cells, glial fibrillary acidic protein, indicating a neuroectodermic origin. However, few glucagon-like peptide-1$^+$ cells did not show coexpression with glial fibrillary acidic protein, suggesting a non-neuroectodermic origin for these cells. Finally, glucagon$^+$ cells did not display Ca^{2+} oscillations typical of pancreatic α-cells. *Discussion.* These results indicate the possible nondefinitive endodermal origin of glucagon-positive cells spontaneously differentiated from D3 and R1 cell lines, as well as the presence of cells expressing glucagon-like peptide-1 from two different origins.

Key Words: *cell culture, definitive endoderm, neuroectoderm, pancreatic hormones, stem cells*

Introduction

In the search for bioengineering protocols to obtain insulin-producing cells from embryonic stem cells (ESCs), many investigators have reported the presence of glucagon$^+$ cells in their cultures (1–9). The expression of glucagon has always been accepted as a definitive endoderm marker. In this sense, glucagon is expressed in several endoderm-derived tissues, such as the endocrine pancreatic α-cells and intestinal L-cells. However, the hypothalamus, thalamus and septal regions of the brain (neuroectoderm-derived tissues) also express glucagon (10,11). Nevertheless, the final hormone product differs in the different cell types: mature glucagon is produced in α-cells, whereas glucagon-like peptide-1 (GLP-1) is found in both L-cells and neuroectoderm-derived tissues (10). Therefore, not all glucagon$^+$ cells obtained in bioengineering protocols necessarily originate from the definitive endoderm, because neuroectoderm-derived glucagon-expressing cells may also be present.

Even though β-cells have always been the center of attention because of their direct implication in diabetes, the roles of the other accompanying cells, in particular α-cells, are beginning to be elucidated (10). Thus, it would be of great interest to study glucagon-expressing cells in bioengineering protocols. It is well known that the different cell types of the pancreatic islet work as a whole, cooperating in the process to control circulating nutrient levels (12). Therefore, bioengineered glucagon$^+$ cells could serve together with glucagonoma cell lines (i.e., α-TC1-9) as cell models to study relevant aspects of the biology of these cells, including stimuli that modulate glucagon secretion.

It is also known that many preproglucagon-derived peptides exert a key incretin role, such as GLP-1, which stimulates β-cell replication and

Correspondence: Dr. **Enrique Roche**, PhD, Instituto de Bioingeniería, Universidad Miguel Hernandez, Avda de la Universidad sn, 03202-Elche (Alicante), Spain. E-mail: eroche@umh.es

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function (13). In this context, glucagon-producing cells have been proposed to create a customized niche that positively favors the survival and function of transplanted insulin-secreting cells (14).

Recently, it has been demonstrated in the adult rodent pancreas that glucagon-expressing cells have a higher plasticity than expected (15). When transgenic mouse islets were completely depleted of β-cells and in the absence of autoimmune and inflammatory responses, remaining glucagon$^+$ cells were capable of transdifferentiating into insulin-secreting cells. In this context, *in vitro* protocols using human cells frequently give rise to cells expressing both hormones (1,16). Furthermore, the co-expression of these hormones is also found in insulinoma cell lines (10). Besides, Pdx-1 downregulation, which decreases insulin gene expression, favors preproglucagon expression (17). These observations indicate that bioengineered glucagon-expressing cells could serve as tools to screen compounds that are capable of transdifferentiating α-cells to β-cells. Finally, ESCs also may serve as a tool to study particular aspects of glucagon$^+$ cells derived from other lineages that are at present poorly characterized, such as neuroectoderm.

Thus, the purpose of this work is to characterize the glucagon$^+$ cells obtained by spontaneous differentiation of ESCs to define their origin by studying their gene expression pattern, protein products and intracellular Ca^{2+} patterns.

Methods

Cell cultures

The D3 mouse cell line was used (American Type Culture Collection, Manassas, VA, USA). Undifferentiated cells were grown in gelatin-coated (0.1% gelatin on 1× phosphate buffered saline; Sigma, St. Louis, MO, USA) dishes (Techno Plastic Products, Trasadingen, Switzerland) in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) containing 4.5 g/L glucose, 15% fetal bovine serum (FBS) (Linus-Cultek, Barcelona, Spain), 1× nonessential amino acids, 100 U/mL penicillin, 100 µg/mL streptomycin (all reagents from Invitrogen) and 1000 U/mL of mouse leukemia inhibitory factor (LIF) (ESGRO, Millipore, Billerica, MA, USA), which was used to maintain D3 cells in an undifferentiated state. The medium was changed daily.

Spontaneous differentiation was carried out by transferring 5×10^4 cells/mL to nonadherent dishes (Deltalab, Barcelona, Spain), in which embryoid bodies (EBs) were formed and then incubated in the absence of LIF for 28 days. At the end of this period, EBs were transferred to adherent plates to allow expansion of committed cells (outgrowth phase). Mouse R1-ESCs were cultured as previously described (18).

α-TC1-9 cells were cultured in DMEM containing 16.7 mmol/L glucose, 10% FBS, 1× nonessential amino acids, 100 U/mL penicillin, 100 µg/mL streptomycin, 10 mmol/L Hepes, pH 7.4 (Invitrogen), 2 mmol/L glutamine (Invitrogen) and 1.5 g/L sodium bicarbonate (Sigma).

Reverse transcription—polymerase chain reaction

For total RNA isolation, the EBs were grown for 28 days in medium without LIF and outgrowths for 7 additional days. Total RNA was extracted using Tripure (Roche, Basel, Switzerland) according to manufacturer instructions. RNA was quantified by the 260/280 nm optical density ratio. One microgram of RNA was reverse-transcribed with the use of Expand Reverse Transcriptase and oligo deoxythymidine primers (Roche). Of the reverse transcription product obtained, 1 µL was used for PCR, using specific primers and the Expand High Fidelity PCR system (Roche). The mixture was first denatured for 5 min at 95°C, followed by the corresponding cycles: 30 sec denaturalization at 94°C, 30 sec at the corresponding annealing temperatures and elongation for 1.5 min at 72°C, plus a final elongation step of 10 min at 72°C. Primer sequences and PCR conditions are indicated in Table I.

Real-time PCR

For quantitative real-time PCR analysis (qPCR), 1 µL of complementary DNA (obtained from retrotranscribed RNA isolated from 28-day EBs and 7-day outgrowths) was amplified by means of the Light Cycler FastStart DNA Master^{PLUS} SYBR Green I kit (Roche). The $2^{(-\Delta\Delta Ct)}$ method (19) was used for the quantification of PCR products using β-actin as the unvariant internal control. The PCR program consisted of an initial denaturing step of 10 min at 95°C, followed by 40 cycles of 10-sec denaturing at 95°C, 7 sec at the corresponding annealing temperatures and 12 sec of elongation at 72°C, plus a final melting curve step. Primer sequences and conditions are indicated in Table I.

Plasmid construction and cell transfection

The 1.6-kb glucagon promoter (from 1.6 Glu-BGL-CRE; B. Laser) was ligated in the SacI (Roche) site of the pCR2.1 vector (Invitrogen, Barcelona, Spain) and the NeoSV40pA cassette (from plasmid containing complementary DNA3.1(+)/CAT; Invitrogen) in the BamHI (Promega, Madison, WI, USA) site after glucagon promoter. The construct also included a phosphoglycerate-kinase-hygromycin resistance (pGK-Hygro-pA) sequence, cloning in the BglII

Table I. Specific PCR primers and conditions used.

Gene	Primer set	Product (bp)	Accession number	Annealing temperature	Cycles
Oct 3/4	Fw: 5′-TTCTGGCGCCGGTTACAGAACCA-3′ Rv: 5′-GACAACAATGAGAACCTTCAGGAGA-3′	271	GI_53500	60°C	30
Amnionless	Fw: 5′ ACTGCCTCCAACTGGAACCAGAAC 3′ Rv: 5′ CGCAGAGGTCACAGCATTGTCCTT 3′	667	BC087954.1	62°C	35
Neurofilament M	Fw: 5′ CTTTCCTGCGGCGTAATCACGAA 3′ Rv: 5′ GGAGTTTCCTGTACGCGGCG 3′	518	NM_008691	63°C	35
Brachyury	Fw: 5′ GCTCATCGGAACAGCTCTCCAACC 3′ Rv: 5′ GGAGAACCAGAAGACGAGGACGTG 3′	319	NM_009309	61°C	30
Flk1/Kdr	Fw: 5′-CAGCCAGACAGACAGTGGGAT-3′ Rv: 5′-CCGAGGCCACAGACTCCCTGCTT-3′	124	NM_010612	63°C	35
Insulin I	Fw: 5′ TAGTGACCAGCTATAATCAGAG 3′ Rv: 5′ ACGCCAAGGTCTGAAGGTCC 3′	288	NM_008386	58°C	38
Insulin II	Fw: 5′ CCCTGCTGGCCCTGCTCTT 3′ Rv: 5′ AGGTCTGAAGGTCACCTGCT 3′	212	NM_008387	60°C	38
Preproglucagon	5′ GAGAGGCATGCTGAAGGGACC 3′ 5′ CATCCCAAGTGACTGGCACGAG 3′	313	NM_008100	62°C	35
PC1	Fw: 5′-AGTGGTGATTACACAGACCA-3′ Rv: 5′-TCCCTTTCAGCCAACAGTAC-3′	146	NM_013628.2	60°C	30
PC2	Fw: 5′-GACGGTGGCAGCTACGATGACTGC-3′ Rv: 5′-CGGAGCAGCTGCAGATGTCCCA-3′	237	NM_008792	62°C	30
PC5	Fw: 5′-GACAAGTGCAGCGGAAAGGAGG-3′ Rv: 5′-ATGCACTCATCTGTGGAACTCTGGC-3′	311	NM_001190483.1	60°C	35
β-Actin	Fw: 5′-TGGGAATGGGTCAGAAGGAC-3′ Rv: 5′-TGAAGCTGTAGCCACGCTCG-3′	468	NM_007393.1	62°C	25
Glucagon[a]	Fw: 5′-GGCTCCTTCTCTGACGAGATGAGCAC-3′ Rv: 5′-CTGGCACGAGATGTTGTGAAGATGG-3′	141	NM_008100	61°C	40
β-Actin[a]	Fw: 5′-CCCTAGGCACCAGGGTGTGA-3′ Rv: 5′-TCCCAGTTGGTAACAATGCCA-3′	128	NM_007393.1	60°C	40

[a]Conditions used in qRT-PCR.

(Roche) site, to allow the selection by hygromicin of transfected cells. The new construct, called Kni 1.0, was 9.1 kb. A total of 15×10^6 D3-ESCs (70% confluence) were electroporated with 25 μg of Hind III (Promega) linearized construct, using an Electro Square Porator ECM830 (BTX, Genetronics, San Diego, CA, USA), applying one pulse of 250 mV for 1 msec, resulting in an efficiency of 30% of transfected cells; 5×10^6 cells/dish were seeded onto 100-mm gelatinized culture dishes. The selection of transfected cells was initiated 24 h after electroporation by adding 200 μg/mL hygromicin (Sigma) to the media. A parallel control of nontransfected cells was incubated in the presence of the antibiotic. Hygromicin selection was stopped when all control cells were dead. This corresponds to the fifth day of hygromicin addition.

Immunocytofluorescence

Antibodies against glucagon and GLP-1 were used. Initially it was necessary to verify that there was no cross-reaction. To this end, antibodies were analyzed by Western blot with the use of α-TC1-9 protein extracts. Glucagon antibody detected two bands coinciding with preproglucagon (21 kDa) and proglucagon (18 kDa) peptides. However, we could not detect processed glucagon. This result could indicate a resolution problem with the technique caused by the low molecular weight of the peptides being analyzed. In support of the latter, we did not find any report in which glucagon had been detected by electrophoresis. On the other hand, GLP-1 antibody was unable to detect glucagon, proglucagon or preproglucagon, coinciding in this sense with manufacturer's indications. Therefore, and according to these observations, for immunocytofluorescence experiments it has to be taken into account that glucagon antibody should be able to detect at least the three related peptides (preproglucagon, proglucagon and glucagon), whereas the GLP-1 antibody appears to be more specific for this particular peptide.

EBs at day 28 were adhered onto 24-mm glass slides treated with 0.1 mg/mL poly-L-lysine, 0.01% fibronectin and 0.2% gelatin (all reagents from Sigma). EBs were grown for 7 days, then the preproglucagon$^+$ cells were selected with 60 μg/mL G-418 (Invitrogen) for 5 days. The cells were treated with 4% paraformaldehyde (Panreac, Barcelona, Spain) for 1 h and then permeabilized with 0.05% Triton and blocked for 1 h with 10% goat serum, 10% rabbit serum and 3% bovine serum albumin (BSA) (all reagents from Sigma) for the neurofilament middle (NefM)/glucagon

inmunocytofluorescence, or 10% goat serum and 3% BSA for 16 h at 4°C for glial fibrillary acidic protein (GFAP)/GLP-1. Afterward, the cells were incubated with the anti-glucagon monoclonal antibody (1:200; Sigma) and an anti-NefM polyclonal antibody (1:500; Stemcell, Grenoble, France) or with an anti-GFAP monoclonal antibody (1:200; Becton-Dickinson, NJ, USA) and the anti-GLP-1 antibody (1:50). After primary antibodies were removed, the cells were incubated for 1 h with the following secondary antibodies: anti-mouse immunoglobulin G (IgG) conjugated with fluorochrome Cy3 (1:1000; Jackson, Suffolk, United Kingdom) for glucagon, Alexa F488 (1:100; Invitrogen) for GFAP, anti-chicken IgY conjugated with fluorochrome fluorescein isothiocyanate (1:500; Abcam, Cambridge, United Kingdom) for NefM and anti-rabbit IgG conjugated with Cy3 (1:500; Becton Dickinson) for GLP-1. An epifluorescence microscope (Olympus AX 70) was used to visualize the cells, and the images were taken with the use of DP-SOFT 3.2 software. To estimate the amount of preproglucagon$^+$ or GLP-1$^+$ cells, we counted fluorescent cells in seven microscope fields from three independent experiments and determined this as a percentage in respect to the total cell number, visualized by transmission.

Recording of intracellular calcium

Neomycin-selected preproglucagon cells derived from EB explants were loaded with 5 μmol/L Fura-2 acetoxymethyl ester for at least 1 h at 37°C and 5% carbon dioxide. Calcium recordings in cells were obtained by visualizing intracellular calcium under a × 60 oil immersion lens in an inverted epifluorescence microscope (Zeiss, Axiovert 200) at 37°C. Images were acquired every 3 sec with an extended Hamamatsu Digital Camera C4742-95 (Hamamatsu Photonics, Barcelona, Spain), using a dual-filter wheel (Sutter Instrument Co, Novato, CA, USA) equipped with 340- and 380-nm, 10-nm bandpass filters (Omega Optics, Madrid, Spain). The data were acquired with the use of ORCA software from Hamamatsu (Hamamatsu Photonics, Barcelona, Spain). Fluorescence changes were expressed as the ratio of fluorescence at 340 nm and 380 nm (F340/F380). Results were plotted with the use of commercially available software (Excel, Microsoft). Changes of $[Ca^{2+}]_i$ oscillations were monitored during a period of 10−15 min with a flow of 1-2 mL/min before stimuli application (control), and the stimulus was added for an additional 10 min. The stimuli used were 0.5 and 25 mmol/L glucose, 10% FBS, 10% BSA (Sigma) and 10% fatty acid−free BSA (Roche).

Statistical analysis

Statistical analysis was carried out using the SPSS-10 software for Windows (IBM, Armonk, NY, USA). Results are expressed as mean ± SEM; values of $P < 0.05$ were considered statistically significant and tested by ANOVA.

Results

Embryonic stem cell differentiation to glucagon-positive cells

First, preproglucagon expression, along with several other genes, was analyzed in EBs after 28 days of spontaneous differentiation. In these cultures, it was initially possible to detect two morphologically different EB populations. The majority of the clusters consisted of compact cell aggregates with a dense cellular core, whereas a smaller population consisted of cystic structures containing a central cavity surrounded by thin cell layers (20). Both EB structures appeared after 15 days of EBs culture. Cell clusters of both populations were manually isolated (Figure 1), cultured separately and analyzed for the expression of several representative genes (Figure 2). Of the list of genes studied, only Oct3/4, a pluripotent stem cell marker (21), was present in undifferentiated D3. In addition, Oct3/4 and Amn (amnionless), a marker for primitive endoderm (22), were expressed in both populations of EBs. NefM, an exclusive marker

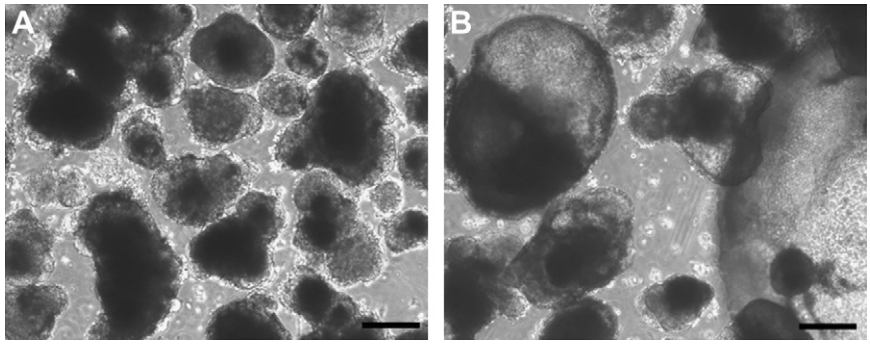

Figure 1. (A) Compact and (B) cystic EBs obtained from D3-ESCs after 28 days of LIF withdrawal. Bar: 100 μm.

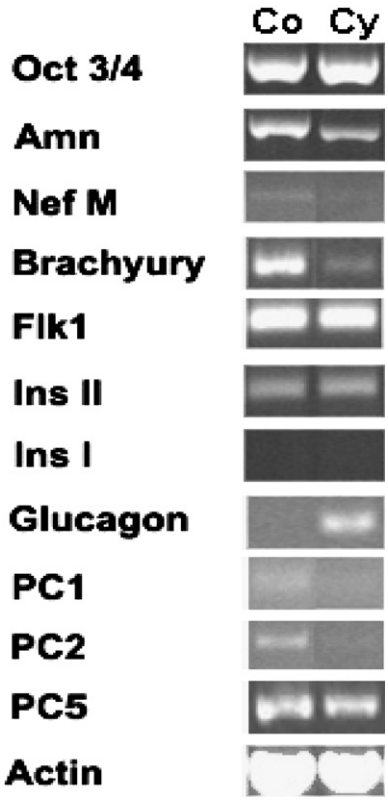

Figure 2. RT-PCR analysis of gene expression after selection of compact (Co) and cystic (Cy) EBs derived from D3-ESCs incubated for 28 days in the absence of LIF.

for neuroectoderm (23), was faintly expressed in compact and cystic EBs. Bry (brachyury), a mesendoderm marker (24) that is generally present in the primitive endoderm (25), was robustly expressed in compact EBs, displaying a weak expression in the cystic population. Flk1/Kdr (fetal liver kinase-1), a mesodermal marker expressed in endothelial precursors (26), was clearly expressed in both populations of EBs. Concerning insulin gene expression, both insulin I and II genes were studied. Insulin II gene was expressed in both cystic and compact EBs, whereas no trace of insulin I expression was observed in either type of EBs. Insulin II is generally expressed in neuroectoderm and primitive endoderm-derived rodent tissues, whereas insulin I is exclusively expressed in definitive endoderm (27), the cell lineage from which pancreatic islets derive. However, in the EBs, insulin I gene was only detected by using qPCR, giving amounts that were one million times lower than the amounts detected in the mouse insulinoma cell line MIN-6 (data not shown). This result indicated that cells of the definitive endoderm lineage were in comparison much less abundant in spontaneous differentiation protocols of ESCs. Finally, preproglucagon gene was only detected in the cystic EB population. Altogether, these results indicate that the spontaneous differentiation in cystic EBs obtained from D3-ESCs gives rise to the two lineages from which the glucagon gene is expressed *in vivo*: neuroectoderm and definitive endoderm (10), although the last one is present clearly in much less proportion.

Regulation of glucagon gene expression in EBs and outgrowths derived from D3-ESCs

To further characterize the spontaneously differentiated glucagon$^+$ cells from D3-ESCs, we analyzed the possible glucose-mediated preproglucagon gene regulation in these cells. Previous results indicate that glucose does not affect preproglucagon messenger (m)RNA levels in rat islets (28). However, the sugar modulates differently, depending on the incubation time, preproglucagon gene expression in α-TC1-6 (29) and α-TC1-9 (30) cells, which are islet cell lines that serve as α-cell models. After a 24-h incubation at high glucose concentrations (16.8–25 mmol/L), preproglucagon mRNA levels were modestly but significantly decreased with respect to the low glucose incubation condition (2.8–5 mmol/L) in both cell lines (29,30). However, long-term (5 days) incubation in hyperglycemic conditions (25 mmol/L) dramatically increased preproglucagon mRNA levels (29).

For this reason, we measured by qPCR the preproglucagon gene expression levels in cystic EBs and outgrowths obtained from spontaneously differentiated D3-ESCs after 24 h in low (5 mmol/L) or high (25 mmol/L) glucose concentrations. First, the relative expression of glucagon in EBs and outgrowths was 10^6 times lower than in α-TC1-9 cells. Second, although preproglucagon mRNA was slightly increased at 5 mmol/L glucose in α-TC1-9 cells, this was not observed in EBs or outgrowths derived from D3-ESCs (Figure 3). This result indicates that preproglucagon gene is not regulated by glucose in the same manner as it was observed in α-TC1-9 cells. This result could suggest that the majority of the stem cell–derived glucagon-expressing cells may derive from the neuroectoderm. However, how the preproglucagon is modulated in neuroectodermal cells is unknown and requires further characterization.

Immunolocalization of preproglucagon and GLP-1 in outgrowths derived from D3-ESCs

Immunocytofluorescence analysis could give additional information concerning the origin of glucagon$^+$ cells spontaneously derived from D3-ESCs by analyzing different protein products derived from the preproglucagon processing. Mature glucagon is produced in α-cells, whereas GLP-1 is found in intestinal L-cells (derived from definitive endoderm) and neuroectoderm-derived tissues (10,13).

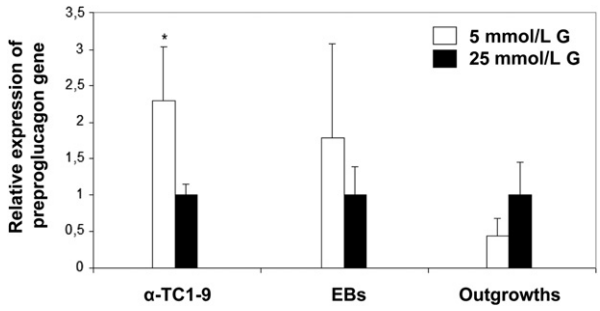

Figure 3. qPCR analysis of glucagon gene expression in α-TC1-9 cells, EBs derived from D3-ESCs after 28 days incubation in the absence of LIF and the corresponding outgrowths after 7 days of expansion in the presence of 5 and 25 mmol/L glucose (G). *Significant differences in respect to 25 mmol/L glucose from four independent experiments, $P < 0.05$.

When glucagon antibody was used in immunocytofluorescence preparations, we observed a very low percentage of positive cells (5% of the total cell number) in outgrowths obtained from spontaneously differentiated D3-ESCs. Besides, when anti-GLP-1 was used in the same type of experiments, the amount of positive cells was even lower (<1% of the total cell number). To circumvent this problem and to obtain a higher amount of glucagon$^+$ cells, we transfected D3-ESCs with a DNA construct including a neomycin resistance under the control of the glucagon gene promoter, allowing for the selection of glucagon-expressing cells by adding G-418 to the culture medium during the outgrowth phase. The neomycin-selected cells were both glucagon and NefM positive (Figure 4A–C). Therefore, and because NefM is an exclusive neuroectoderm marker, this observation strongly suggests that the glucagon-expressing cells were mainly derived from the neuroectoderm. In addition, and taking in account the cross-reactivity of the antibody, we cannot discard that glucagon$^+$ cells were also expressing preproglucagon or even proglucagon. However, the constitutive expression of the PC5 (Figure 2), a prohormone convertase capable of processing preproglucagon to glucagon and GLP-1 (31), strongly suggests the presence of both peptides in spontaneously differentiated D3-EBs.

Concerning GLP-1, we could not investigate the co-expression of this peptide with NefM because of the coincidence of secondary antibodies. Therefore, we decided to study the co-expression of GLP-1 with GFAP, a protein that is also well expressed in neuroectoderm. As expected, the majority of the neomycin-selected cells express GFAP (Figure 4E), according to their neuroectodermal origin. However, we have observed two cell types, those that only express GLP-1$^+$ and cells that co-express GLP-1 and GFAP (Figure 4F,G). Because the GLP-1 antibody does not detect other peptidic forms, such as preproglucagon, we can only assume that the immunopositive cells expressed this peptide.

To rule out that these observations could be stem cell line–specific, we repeated some of the experiments with R1-cells, another mouse ESC line. EBs derived from R1-ESCs presented an expression pattern very similar to those observed in D3, although glucagon expression was evident at day 21 (18). With respect to the immunocytochemistry experiments, R1 coexpressed preproglucagon and NefM and GLP-1 and GFAP as well. Besides, few GLP-1$^+$ cells did not show positive GFAP-label (Figure 4I–K). Altogether, we can conclude that in both ESC lines (R1 and D3), the majority of the glucagon- and GLP-1–expressing cells have a neuroectodermal origin; however, a very low percentage of cells expressing GLP-1 appears to derive from a different lineage that remains to be identified.

Analyses of Ca^{2+} patterns in glucagon-positive cells derived from D3-ESCs

To further characterize the glucagon$^+$ cells, functional analyses regarding intracellular calcium oscillations were performed. Total glucagon content could not be determined because of the low number of glucagon$^+$ cells obtained after neomycin selection. Therefore, we decided to measure intracellular Ca^{2+} patterns, which are a characteristic feature of glucagon-secreting cells (12). The tumoral cell line α-TC1-9 was used as reference control because this cell line presents Ca^{2+} patterns similar to pancreatic α-cells (30), remaining silent at high extracellular glucose concentrations (25 mmol/L) and presenting Ca^{2+} oscillations at low glucose concentrations (0.5 mmol/L) (Figure 5). However, 100% of the glucagon$^+$ cells obtained after neomycin selection did not show Ca^{2+} changes at low or high glucose concentrations (Figure 5). The 53% of selected glucagon$^+$ cells only responded to factors present in FBS; this observation was also seen in α-TC1-9 cells (Figure 5). Furthermore, the glucagon-selected cells obtained in our protocols did not respond to fatty acids bound to BSA, an exclusive stimulus of fetal astrocytes (32). As expected, this response was also absent in α-TC1-9 cells (Figure 5). Therefore, and from a functional point of view, the studied glucagon$^+$ cells obtained from ESCs do not display the intracellular Ca^{2+} pattern that is typical of pancreatic α-cells.

Discussion

We have previously demonstrated that insulin is expressed in the neuroectoderm as well as in primitive and definitive endoderm-differentiated ESCs

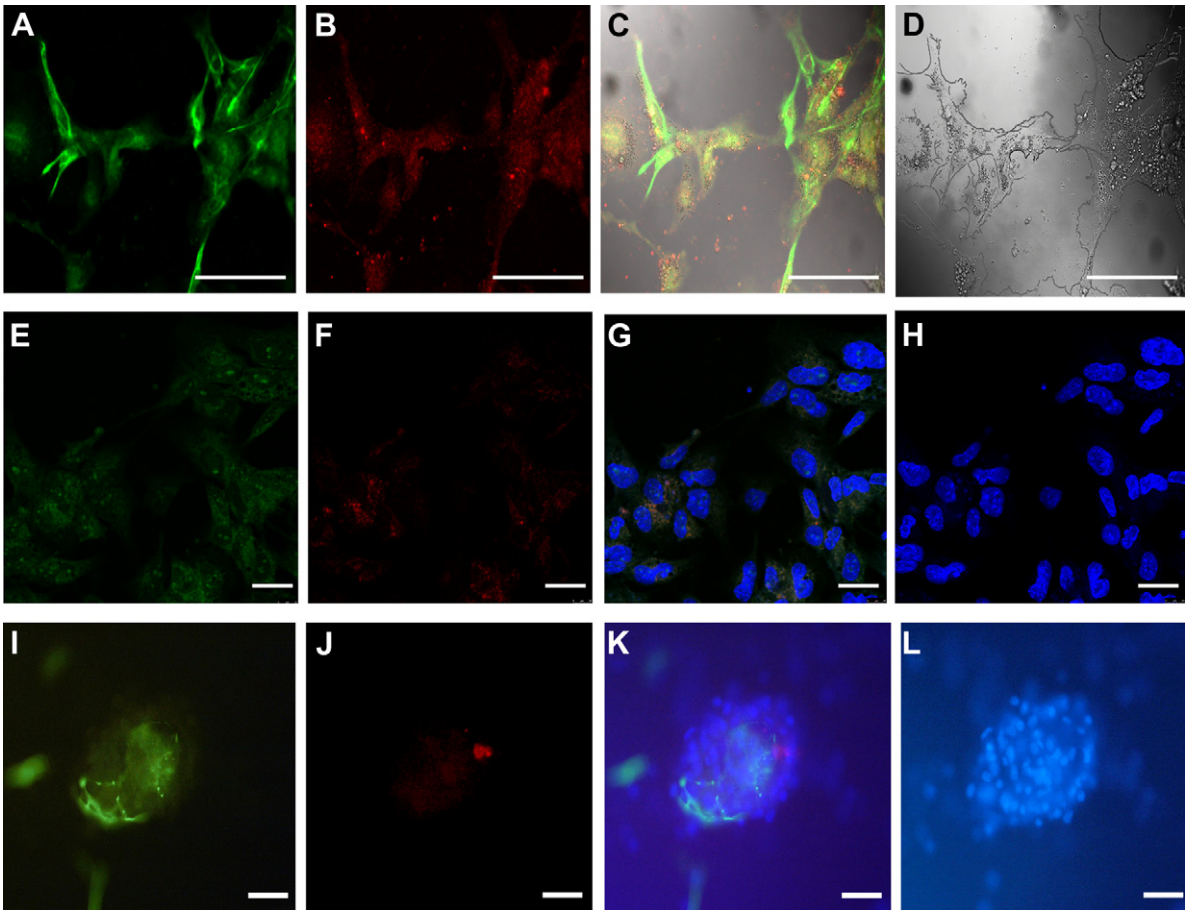

Figure 4. Representative immunocytofluorescence for (A) NefM and (B) glucagon in neomycin-selected outgrowths of D3-ESCs (see Methods). (D) Transmission image is shown as a control of cell number. (C) Merged image from A, B and D. Representative immunofluorescence for (E) GFAP and (F) GLP-1 in outgrowths of D3-ESCs. (H) Hoechst staining is shown as a control of cell number. (G) Merged image from E, F and H. Representative immunocytofluorescence for (I) GFAP and (J) GLP-1 in outgrowths of R1-ESCs. (L) Hoechst staining is shown as a control of cell number. (K) Merged image from I, J and L. See the text for more details. Bars: 100 μm (A–H) and 250 μm (I–L).

in vitro (33), coinciding with *in vivo* observations (34). In this context, and because glucagon is an important partner of insulin in almost all bioengineering protocols (1–9), we decided to analyze the possible origin of glucagon-expressing cells obtained from ESCs by spontaneous differentiation.

Glucagon is the counterpart of insulin in the islets of Langerhans, being released in hypoglycemic conditions to provoke hepatic glucose production (10). In this context, the control of preproglucagon biosynthesis, processing and glucagon secretion is under the control of several stimuli, which include nutrients, paracrine hormones released by neighboring β- and δ-cells and neurotransmitters of the autonomic nervous system (10). In addition, abnormal glucagon secretion is emerging as a key hormonal disruption that accompanies diabetes mellitus anomalies (10,35).

The particular architecture of pancreatic islets is instrumental for the control of the large variety of extracellular signals that fine-tune hormonal secretion under different physiopathological situations. Therefore, the key objective in cell therapy for diabetes might be to mimic and preserve intra-islet organization to optimize the endocrine function, where α-cells play a key role (36). In addition, it has been demonstrated recently that glucagon$^+$ cells are involved in restoring new β-cell mass in depleted islets (15). These observations indicate that glucagon-expressing cells deserve more attention in future cell therapy protocols to treat diabetes.

At present, there is no information concerning glucagon$^+$ cells that appear in ESCs during the spontaneous differentiation protocols (18). The presence of preproglucagon gene expression has been noticed in protocols aiming to obtain insulin-producing cells from ESCs (1–9), but the cells expressing glucagon have not been studied in detail. Recently, glucagon-secreting α-cells have been obtained and deeply characterized in an *in vitro* coaxial

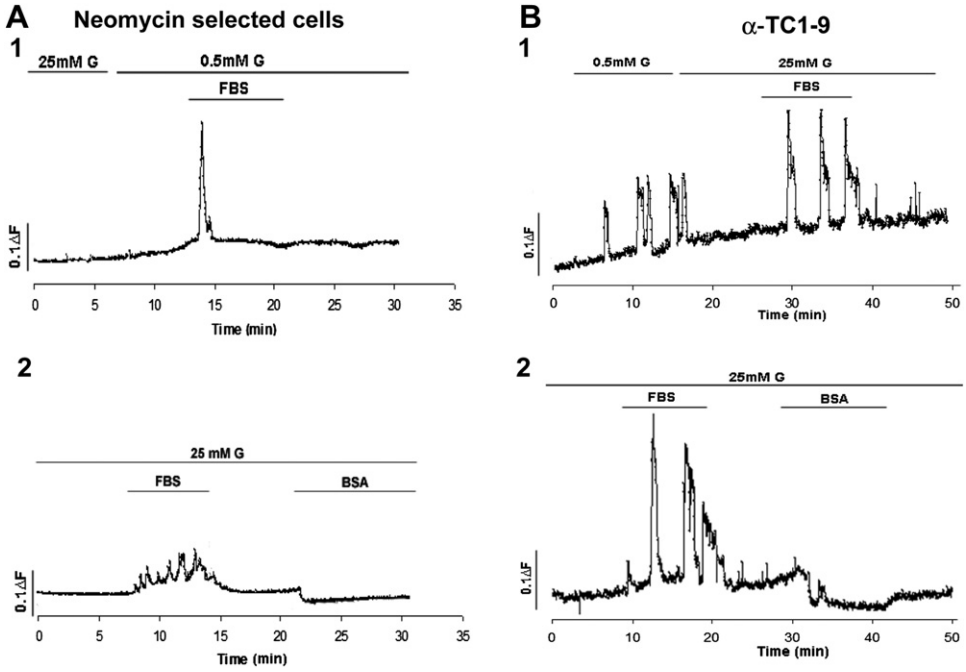

Figure 5. Intracellular Ca^{2+} patterns of outgrowths derived from neomycin-selected D3-ESCs (A1, n = 4; A2, n = 18) and in α-TC1-9 cells (B1, n = 39; B2, n = 16) incubated in the presence of 0.5 mmol/L glucose (G), 25 mmol/L glucose, 15% FBS and 10% BSA.

protocol from human ESCs (36). In this publication, the cells underwent a staged protocol in which cells committed to mesendoderm evolved to immature precursors coexpressing insulin and glucagon until reaching a mature state in which only glucagon was expressed. The resulting cells were similar to pancreatic glucagon-secreting cells *in vivo* and expressed specific transcription factors of this cell type such as ARX. However, in our report the glucagon$^+$ cells spontaneously obtained after EB formation were of a neuroectodermal origin, according to the markers expressed, and were ARX negative (data not shown). In addition, and taking into account the high constitutive expression of PC5 instead of PC1 and PC2 (Figure 2), we hypothesize that the majority of the protein found should correspond to glucagon and GLP-1.

Very small numbers of GLP-1$^+$ cells were detected. GLP-1 appears to be expressed in cells that derive as well as in cells that do not derive from neuroectoderm. One possibility is that these cells derive from definitive endoderm precursors, but this lineage is extremely scarce in spontaneous differentiation protocols, as indicated by the minute amounts of insulin I expression detected.

The glucagon$^+$ cells that we have obtained by spontaneous differentiation differ from other neuroectodermal insulin$^+$ cells spontaneously obtained *in vitro* in our laboratory. For example, neuroectodermal insulin-expressing cells can be quickly (2–3 days) obtained in monolayers of D3 and R1 cultures in the absence of LIF, whereas glucagon expression requires long-term EBs cultures (>20 days). Neuroectodermic insulin$^+$ cells exhibited Ca^{2+} oscillations typical of immature neuroectodermal cells such as fetal astrocytes (33), whereas the glucagon$^+$ cells described in this report did not respond to fatty acids bound to BSA or KCl (not shown).

Finally, the very scarce amount of glucagon$^+$ cells limits quantification of the hormone by radioimmunoassay techniques, the gold standard functional assay. Taking into account data provided by other authors (12,36) and the detection limit of the commercial kit, we can estimate that the total glucagon content present in our cells is <20 pg/1000 cells, which represents approximately 2–3% of islet content. Further maturation strategies must be implemented *in vitro* to increase the number of glucagon-expressing cells, allowing a more detailed phenotypic characterization.

In conclusion, glucagon$^+$ cells can be obtained spontaneously *in vitro* from D3 and R1-ESCs and are mainly of neuroectodermic origin. A minor percentage of glucagon$^+$ cells (<1%) producing GLP-1 appears to have two different origins (neuroectodermic and non-neuroectodermic). However, the extremely limited numbers of GLP-1$^+$ cells obtained make it very difficult to assess their real origin. Altogether, the cell system presented in this work may represent an alternative *in vitro* model to study preproglucagon gene expression in a neuroectodermic context.

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