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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