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Ultrastructural and molecular analyzes of insulin-producing cells induced from human hepatoma cells

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Abstract

Background aims. Diabetes type I is an autoimmune disease characterized by the destruction of pancreatic insulin-producing (beta-) cells and resulting in external insulin dependence for life. Islet transplantation represents a potential treatment for diabetes but there is currently a shortage of suitable organs donors. To augment the supply of donors, different strategies are required to provide a potential source of beta-cells. These sources include embryonic and adult stem cells as well as differentiated cell types. The main goal of this study was to induce the transdifferentiation (or conversion of one type cell to another) of human hepatoma cells (HepG2 cells) to insulin-expressing cells based on the exposure of HepG2 cells to an extract of rat insulinoma cells (RIN). *Methods.* HepG2 cells were first transiently permeabilized with Streptolysin O and then exposed to a cell extract obtained from RIN cells. Following transient exposure to the RIN extract, the HepG2 cells were cultured for 3 weeks. *Results.* Acquisition of the insulin-producing cell phenotype was determined on the basis of (i) morphologic and (ii) ultrastructural observations, (iii) immunologic detection and (iv) reverse transcription (RT)-polymerase chain reaction (PCR) analysis. *Conclusions.* This study supports the use of cell extract as a feasible method for achieve transdifferentiation of hepatic cells to insulin-producing cells.

Keywords: *beta-cells, diabetes, insulin-producing cells, transdifferentiation*

Introduction

Diabetes is one of the most prevalent diseases worldwide, affecting approximately 6–8% of the world population, and the number of newly diagnosed patients is increasing yearly (1). It has been estimated that 171 million people were affected globally by diabetes in 2000, and this number is expected to rise to 366 million people by 2030 (2).

Type I diabetes is an autoimmune disease involving destruction of the pancreatic insulin-producing cells (beta-cells), resulting in external insulin dependence for life. The classic treatment for type I diabetes, subcutaneous insulin injections, improves blood glucose levels but euglycemia is not achieved, suggesting an essential role of glucose sensing and fine regulation of insulin secretion in beta-cells (3,4). In this context, cell replacement therapy, involving the use of beta-cells from different sources, is considered a feasible alternative for the treatment of type I diabetes.

The successful outcome of islet transplantation therapy trials (5–7) implies that diabetes may be cured by replenishment of deficient beta-cells. Indeed, advances in islet transplantation procedures have improved the blood glucose levels and insulin requirements of diabetic patients. However, the protocols include immunosuppression regimens that could give rise to adverse effects on graft function (4). Furthermore, islet transplantation protocols normally require more than one donor to reach normoglycemia. It is the limited availability of donor islets that has restricted the widespread application of this approach (8). Consequently, there is intense interest in developing alternative sources of beta-like cells. Sources of *de nono* beta-cells currently being considered include the following. (i) Derivation of

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insulin-producing beta-like cells from human embryonic stem cells (9); however, these cells do not fully differentiate in culture and the use of human embryonic stem cells is still limited by legal and ethical concerns (10). These limitations could be overcome by (ii) combining cell dedifferentiation into pluripotency (iPS cells) and directed differentiation (4), although the use of integrating lentiviral and retroviral vectors and the expression of the proto-oncogene c-Myc limits this approach. Another interesting alternative is based on (iii) using a patient's own pancreatic cells, as some evidence of innate beta-cell regeneration by means of replication, even in patients with long-standing diabetes type I, has been described (11–13). However, elucidation of the factors involved in the replication capacity of beta-cells is needed in order to exploit this potential. Finally, there is the phenomenon of (iv) transdifferentiation, the conversion of one differentiated cell type to another (14). The change in cell phenotype of differentiated cells could potentially allow transplantation of long-term insulin-producing cells derived from a patient's own cells (15).

There are different strategies for achieving adult cell reprogramming or transdifferentiation: somatic nuclear transfer (16,17), cell fusion (18,19), ectopic expression of master switch genes (20) and cell extract-based methods (21,22). The main goal of the present study was to induce transdifferentiation of human hepatoma cells (HepG2) to insulin-secreting cells by exposing human liver cells to a cell extract from rat insulinoma cells (RIN). These cells lines were chosen on the basis of the relationship that exists between liver and pancreas during embryonic development. Both liver and pancreas arise during development from adjacent regions of the anterior endoderm (23), and presumably differ in the expression of one or a few key transcription factors (master switch genes) (24). We provide evidence, based on ultra-structural characterization, immunologic detection and reverse transcription (RT)-polymerase chain reaction (PCR) analysis, that transient exposition of HepG2 cells to RIN extract is able to reprogram a hepatoma cell line to insulin-producing cells.

Methods

Cells

HepG2 cells were cultured in Dulbecco's modified Eagle's medium–low glucose (DMEM-LG; Sigma, St Louis, MO, USA). RIN cells were cultured in Roswell Park Memorial Institute (RPMI-1640; Sigma). Both media were supplemented with 10% fetal bovine serum (FBS; Sigma), 100 IU/mL penicillin/100 µg/mL streptomycin (Invitrogen, Merelbeke, Belgium) and 2 mM L-glutamine (Sigma). HepG2 medium was also supplemented with 1% non-essential amino acids (Sigma).

Preparation of RIN cell extract and exposure to HepG2 cells

Cell permeabilization and extract preparation were performed as described elsewhere (21). Briefly, RIN cells (35 000 000–40 000 000) were harvested by trypsin digestion and washed twice in cold phosphate-buffered saline (PBS) and then in cold cell lysis buffer (50 mM NaCl, 5 mM $MgCl_2$, 100 mM HEPES, pH 8.2, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride; Sigma). Cells were centrifuged at 800 ***g***, resuspended in 1.5-volumes of cell lysis buffer containing protease inhibitor cocktail (Sigma) and allowed to swell on ice for 45 min. Cells were homogenized by pulse-sonication and the lysate was sedimented at 15 000 ***g*** for 15 min at 4°C. The supernatant was collected and used fresh. The protein concentration of the extract ranged from 2.31 to 2.54 mg/mL. The extract pH ranged from 7.5 to 8.0. For exposure to cell extract, HepG2 cells were harvested by trypsin digestion and washed twice in ice-cold PBS and once in ice-cold Hanks balanced salt solution (HBSS; Gibco-BRL, Paisley, UK). Cells were pelleted at 500 000 cells/reaction in 1.5-mL tubes and suspended in 488 µL ice-cold HBSS, placed in a water bath at 37°C for 2 min and a final concentration of 230 ng/mL Streptolysin O (SLO; Sigma) added. Samples were incubated for 30 min at 37°C. Cells were centrifuged at 300 ***g*** for 5 min at 4°C in a swing-out rotor. The supernatant was removed and 500 µL extract containing 20 µL 1 mmol/L of each nucleotide triphosphate set (Roche, Indianapolis, IN, USA) added. Cells were incubated with the extracts for 1 h at 37°C. Following incubation, RPMI-1640/10% FCS containing 2 mmol/L $CaCl_2$ was added and 125 000 cells were transferred to 35-mm dishes containing coverslips. After 5 h, dead (floating) cells and the Ca^{2+}-containing medium was replaced with fresh DMEM-LG containing 10% FCS and antibiotics as described above. Cells were cultured for 12–15 days until used. Control cells were either non-permeabilized and non-extract exposed cells or permeabilized cells not exposed to the extracts. Experiments were repeated at least three times, with similar results.

Scanning electron microscopy

Cells were washed three times with PBS and fixed with 2.5% paraformaldehyde (Sigma) in 0.1 M cacodylate buffer (Sigma) for 1 h at room temperature. Cells were post-fixed in 1% osmium tetroxide (Sigma) in aqueous solution in the dark at 4°C

for 1 h and then at room temperature for 1 h. The coverslips were washed three times with distilled water for 10 min and then dehydrated using a gradient of 50%, 70%, 90% and (3×) 100% ethanol. Samples were dried with CO_2 in a Polaron CPD 7501 critical-point drier and finally carbon-covered. Samples were examined with a Carl Zeiss DSM 950 scanning electron microscope (SEM).

Transmission electron microscopy

Cells were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4; Sigma) for 1 h at room temperature and post-fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer (Sigma) for 1 h at room temperature, and dehydrated in ethanol. Cells were detached from the culture vessel with propylene oxide and embedded in Epon 812. After polymerization, the plastic was removed and ultrathin sections were cut parallel and perpendicular to the surface of the flask. The sections were contrasted with uranyl acetate–lead citrate and examined with a Carl Zeiss EM 902 transmission electron microscope (TEM).

Immunofluorescence

Cells were washed three times with PBS, fixed with 4% paraformaldehyde in PBS for 30 min at room temperature, permeabilized with 0.1% Triton X-100 (Sigma) for 15 min, washed three times with PBS and blocked in 2% blocking buffer solution (Roche) for 1 h at room temperature. Cells were then incubated overnight in primary antibody diluted 1:100 in blocking buffer solution at 4°C, followed by washing three times in PBS and then incubating for 2 h with secondary antibodies diluted 1:200 in blocking buffer solution. Samples were washed three times in PBS and coverslips were mounted on slides with DAPI-containing mounting solution (Ultra Cruz Mounting Medium; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Controls were performed with non-treated cells. The primary antibody used was guinea-pig anti-insulin whole antiserum (Sigma). The secondary antibody was Texas Red-conjugated goat polyclonal anti-guinea pig IgG antibody (Abcam, Cambridge, UK).

RT-PCR

For RT-PCR analysis of insulin gene expression, total RNA from treated and non-treated cells was extracted by TRI Reagent® (Sigma) according to the manufacturer's instructions. RNA quality and concentration were assessed using an Ultraspec 2000 UV/visible spectrophotometer (Amersham Pharmacia Biotech, Amersham, UK) and checked on a 1% agarose gel.

The cDNA reaction was performed using 1.5 µg total RNA with primers from an RT system (Promega, Madison, WI, USA) in a Mastercycler ep gradient S thermal cycler (Eppendorf, Westbury, USA), according to the manufacturer's instructions.

The forward and reverse sequences and expected product size for each specific primers were as follows: for human insulin, 5′-GGGGAAC GAGGCTTCTTCTA-3′ and 5′-AGAGGGAG CAGATGCTGGTA-3′ (178 bp); for rat insulin I, 5′-CTACCATCATAGACCATCAGCA-3′ and 5′-CAGTTGGTAGAGGGAGCAGAT-3′ (356 bp); for β-actin, 5′-ATCATGTTTGAGACCTTCAA-3′ and 5′-CATCTCTTGCTCGAAGTCCA-3′ (316 bp). The PCR reaction was performed with GoTaq® green master mix (Promega); after initial denaturation (5 min at 94°C), 35 cycles were performed (20 s at 94°C, then 20 s at 52°C for human insulin, 49.5°C for rat insulin and 45°C for β-actin, and finally 1 min at 72°C) and amplification products were checked by 1% agarose gel electrophoresis using a Hyperladder™ I (Bioline, London, UK).

Results

Morphologic changes associated with reprogramming

The morphologic changes associated with HepG2 cells exposed to RIN cell extract were analyzed by optical microscopy. Control RIN cells expressed a typical neuronal-like morphology with small bodies and tiny braches (Figure 1a), in contrast to control HepG2 cells which were flatter and did not possess any of the neuronal extensions (Figure 1b). HepG2 cells exposed to the RIN cell extract and maintained for 3 weeks in culture displayed a mixed morphology, some were morphologically very similar to RIN cells (black arrows) and some maintained the morphologic features of the original HepG2 cells (white arrow) (Figure 1c). On the other hand, control HepG2 that had been permeabilized but not exposed to RIN cells extract retained the normal morphology of control HepG2 cells (Figure 1d).

TEM

Ultrastructural analysis of cells was performed and compared with non-treated HepG2 and RIN cells. Transdifferentiated cells showed typical ultrastructural features of insulin-secreting RIN cells. Thus these cells had electron-dense nuclei and the cytoplasm occupied large areas and contained increased numbers of organelles, including polyribosomes and rough endoplasmic reticulum. A large number of elongated mitochondria with clear matrices

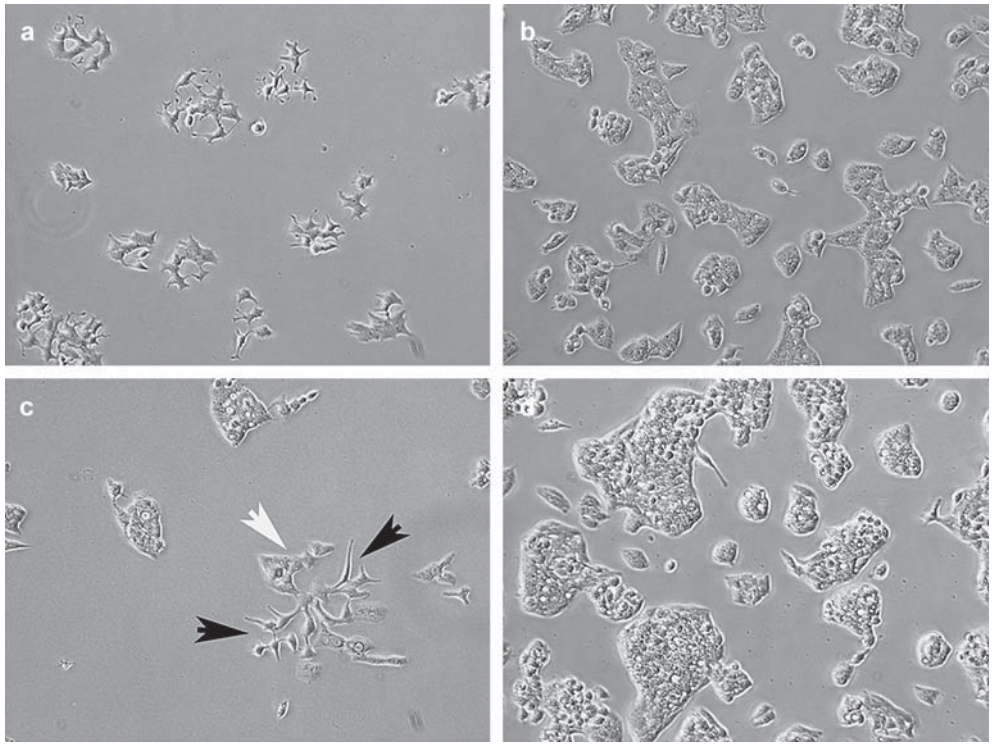

Figure 1. Morphologic changes of HepG2 cells exposed to RIN extract. (a) Control RIN cells; (b) control HepG2 cells; (c) treated cells showing two populations, cells that have acquired morphologic characteristics of RIN cells (blacks arrows) and cells that retain the form of the original HepG2 cells (white arrow). (d) HepG2 cells that were permeabilized but not exposed to the RIN extract. Original magnification 20×.

and dilated cristae were visible. Moreover, the most characteristic feature was the presence of insulin secretory granules, small rounded-shaped vesicles of variable size with a high electron density core and clear halo, distributed mostly in the cell periphery (Figure 2c, d). These structures were also found in RIN cells (Figure 2a) next to plasma membrane, but not in control HepG2 cells (Figure 2b).

SEM

RIN cell examination by SEM showed their typical morphology of cellular prolongations adhered to the substrate with a smooth surface and the distinctive presence of many rounded vesicles and protuberances (Figure 3a, b). Some of these vesicles appeared to have exploded and were expelling theirs contents to the medium (Figure 3d). In contrast, HepG2 cells did not present cell prolongations and showed an irregular cytoplasm membrane with increased numbers of filopodia along the surface, but without vesicles (Figure 3d–f). Interestingly, transdifferentiated cells showed the same round protrusions found in RIN cells but with a rougher surface, as for HepG2 cells (Figure 3h, i). Holes from erupted vesicles were also observed in transdifferentiated cells (Figure 3j).

Insulin expression by immunocytochemistry

Figure 4 shows transdifferentiated beta-like cells and control cells stained and visualized for anti-insulin antibody. Insulin was expressed in a subpopulation of treated cells (Figure 4a). The merge image between contrast–phase microscopy and fluorescence microscopy helped to identify insulin-labeled cells (Figure 4c). Immunocytochemistry showed that treated cells that had acquired an RIN-like morphology (transdifferentiated) exhibited a strong labeling for intracellular insulin (white arrows). However, treated cells that retained the HepG2-like morphology did not express insulin (Figure 4c). Insulin expression was detected in control RIN cells (Figure 4d) but not in HepG2 cells (neither control or permeabilized but not exposed to RIN cell extract) (data not shown).

Insulin expression by RT-PCR

Finally, insulin expression was confirmed further by RT-PCR analyzes. Treated and control cells were tested for insulin expression. Amplification reactions were performed using specific primers for both human and rat insulin, in order to determine whether endogenous insulin was produced in transdifferentiated cells. The gene expression analysis

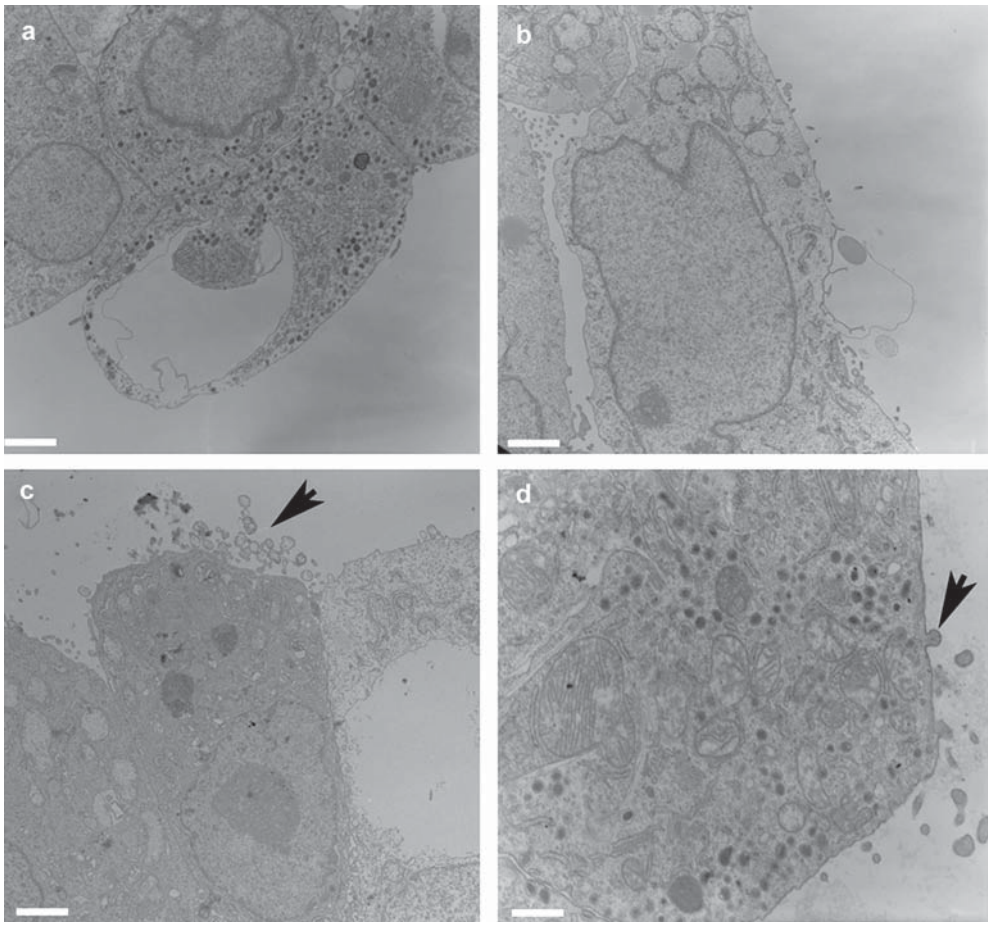

Figure 2. TEM of control (a) RIN cells and (b) HepG2 cells, and (c, d) treated cells showing secretory vesicles (black arrows). Bars: (a, b, c) 1 μm; (d) 400 nm.

demonstrated that control HepG2 cells, as expected, did not show either human or rat insulin expression (data not shown); RIN cells showed rat insulin (Figure 4f) and, finally, only human insulin, and not rat insulin, expression was found in transdifferentiated cells after 3 weeks of culture (Figure 4e). Insulin expression in transdifferentiated cells was lower than in control RIN cells.

Discussion

The number of diabetic patients world-wide is approximately 180 million and this number is expected to double over the next 25 years (25). For serious cases of type 1 diabetes there is an existing method of cell therapy: islet transplantation, in which islets are isolated from an organ donor and grafted into the liver of the patient via the portal vein. The major limitation to islet transplantation is the shortage of organ donors. The ultimate objective of therapy for type 1 diabetes is therefore to replace the functioning beta-cell complement of the body. Thus new sources of beta-like cells are needed to meet demand.

In the present study, we report the acquisition of beta-cell-like characteristics by the HepG2 cell line after being exposed to a cell extract obtained from RIN cells. The HepG2 cell line is a widely recognized model for hepatic studies that has been proved to express some differentiated hepatic cell markers and have a high proliferation rate (23). This cell line overcomes problems with the use of isolated hepatocytes, such as difficulty in isolation, expansion and propagation (24). RIN cells have been described as a beta-cell model based on their similar characteristics to native beta-cells (26). While some differences, such as rough endoplasmic reticulum abundance and size and electron-dense insulin granules, have been found between RIN and native beta-cells, these cells possess normal morphology and are able to produce and store insulin (27).

Previously, it has been shown that supplementation of the cell culture medium with high concentrations of glucose induces liver-derived cells to differentiate fully to insulin-producing cells by activating late-stage pancreas development genes and genes related to beta-cell function (28,29).

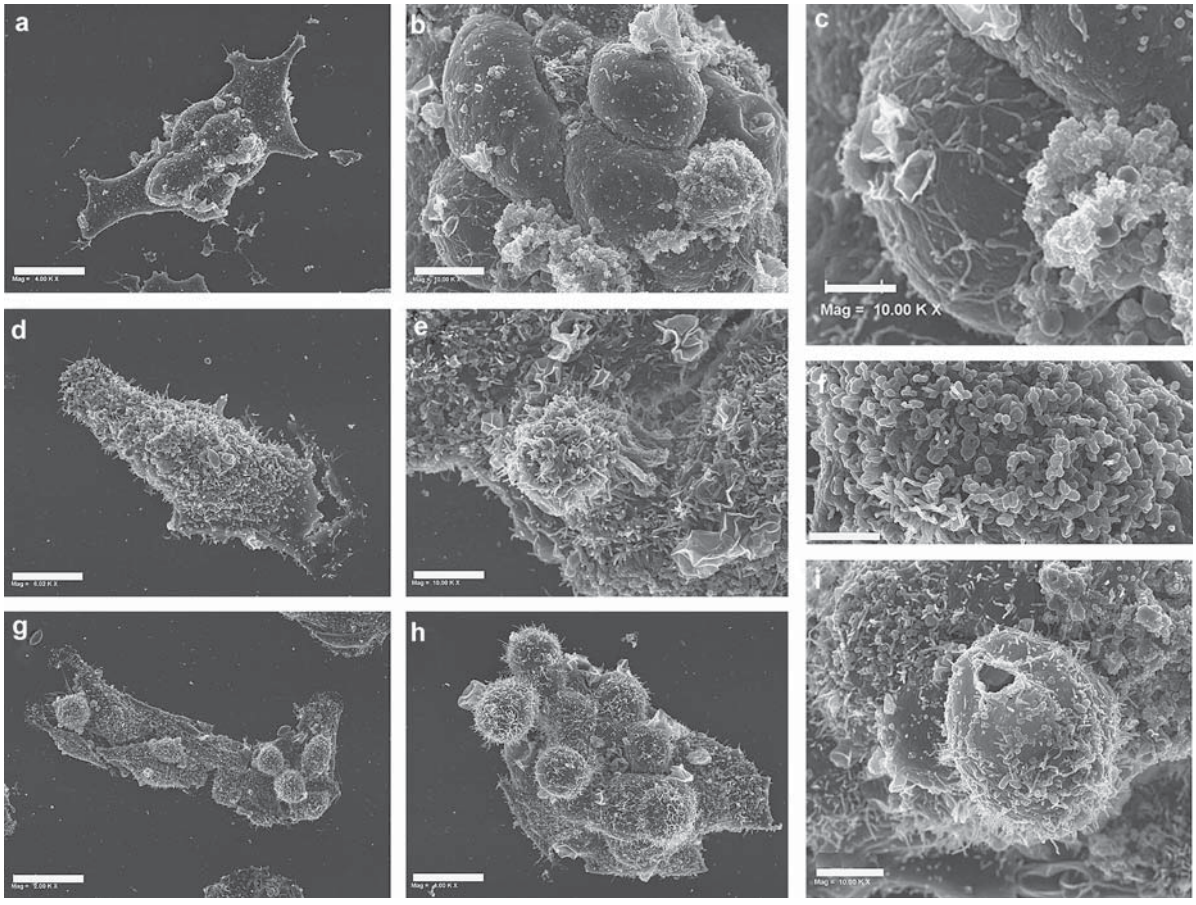

Figure 3. SEM of (a–c) control RIN cells, (d–f) control HepG2 cells and (g–i) HepG2 cells exposed to RIN extract. Bars: (a) 13.5 μm; (b, e, i) 6 μm; (c) 2 μm; (d) 9 μm; (g) 30 μm; (h) 8 μm.

Nevertheless, we provide evidence that extract-exposed cells that were cultured in a low-glucose supplemented medium (1 g/L), displayed morphologic changes at both the structural and ultrastructural levels similar to normal beta-cells. These results demonstrate that the commitment of HepG2 cells towards a new cell fate is independent of glucose stimulation. Transdifferentiated cells presented a RIN-ike morphology, while cells not exposed to extract showed a clear hepatoma phenotype. In addition, electron microscopy showed that reprogrammed cells contained insulin secretory granules, mostly in the cell periphery, as has been shown previously (27,28,30), which suggests that transdifferentiated cells are able to produce and process insulin and form hormone secretory granules (28). These findings were corroborated by inmunocytochemistry and at the gene expression level by RT-PCR analyzes, which showed human insulin expression only in those cells exposed to the RIN extract. Because expression was lower than found in RIN cells, it indicates that not all the treated cells were completely reprogrammed and that induced beta-cell differentiation is a gradual process (28). SEM observations reported some cells showing an RIN-like phenotype and other cells with mixed surface characteristics of both HepG2 and RIN cells. This suggests that reprogramming does not occur at the same time for all cells and, furthermore, involves intermediate phenotypes. In fact, it has been proposed that down-regulation of hepatic genes and activation of beta-cell genes are gradual and parallel in time, co-existing and leading to mixed phenotypes (14).

The cellular commitment may be a consequence of the combination of certain transcription factors. In some cases, there are main factors directing cell differentiation, but some other 'minor factors' are needed for complete differentiation (14). These main factors are known as master switch genes and are important in the development of tissues, because they can lead to a differential development of closely related organs, determining the final fate of cells arising from the same embryonic germ layer (23,24). Pancreatic and duodenal homeobox 1 (Pdx-1) is actually considered to be a master switch gene in pancreatic development, as demonstrated by knock-out (31) and mutation (32) of this gene, leading to arrested pancreatic development and even agenesis,

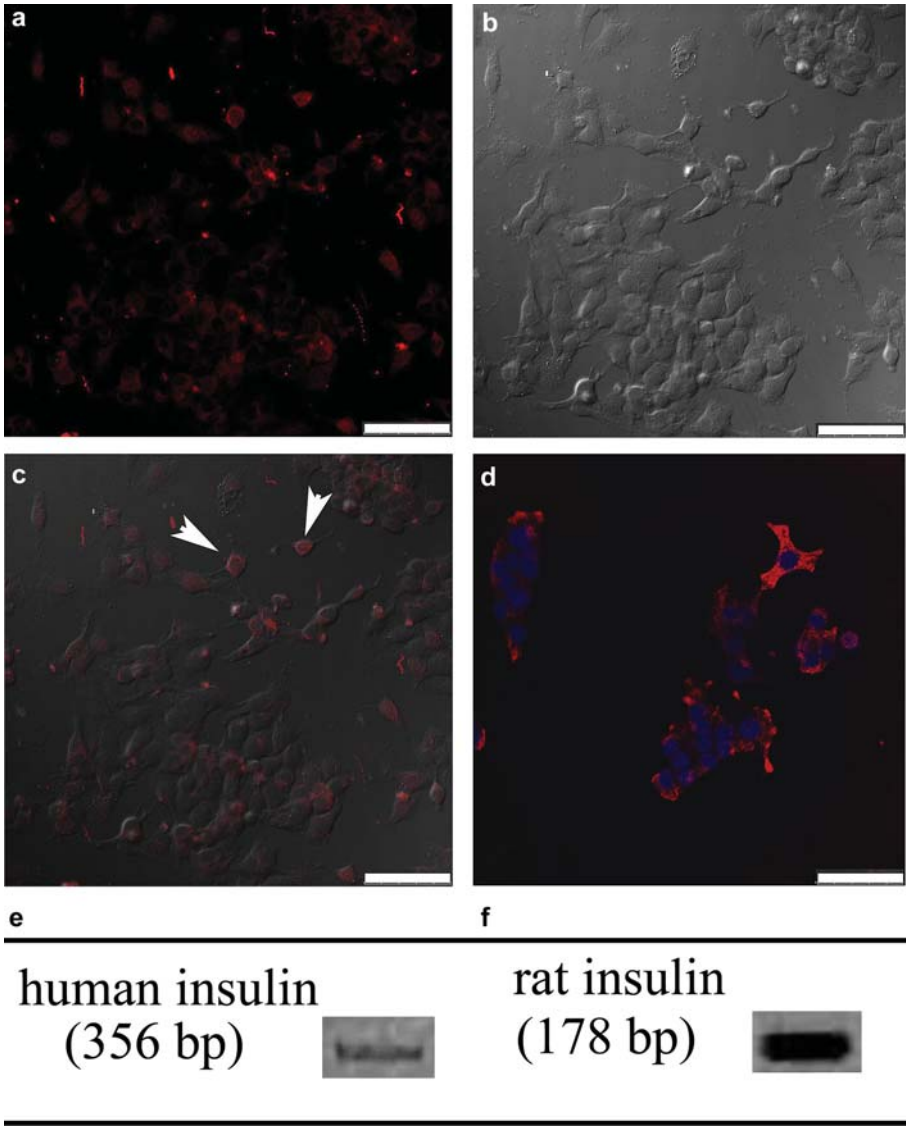

Figure 4. Inmunofluorescence and RT-PCR data showing endogenous expression of insulin in transdifferentiated cells. (a–c) Confocal fluorescence micrograph of treated cells stained for anti-insulin antibody, visualized with a Texas Red secondary antibody (a); (b) the same cells visualized in contrast–phase; (c) a merged image of (a) and (b), two cells with RIN-like morphology showing strong labelling for insulin (white arrows). (d) Labeled control RIN cells. Expression of insulin determined by RT-PCR of (e) treated cells and (f) control RIN cells. Bars: (a–c) 75 μm; (d) 30 μm.

respectively. Pdx-1 efficacy has been demonstrated in many transdifferentiation assays by means of transfection in hepatic cells (24,23) and, in some cases, transplantation of these cells have reversed diabetes in mice (28,33). Although Pdx-1 is a key factor in transdifferentiation from liver to pancreas, additional transcription factors and chromatin remodeling are needed (21). We provide evidence that the extract of RIN cells contains the appropriate combination of main and minor factors to induce cell transdifferentiation of hepatic cells towards a beta-like phenotype. Indeed, a role for accessory transcription factors has been demonstrated, as Pdx-1 is able to initiate differentiation of hepatic cells to both endocrine and exocrine pancreatic cells but requires the participation of other transcription factors in order to induce differentiation towards endocrine cells (28). Thus the activation of Pdx-1 would allow the sequential activation of genes involved in cellular commitment to, and survival and function of, beta-cells. Simultaneous ectopic expression of Pdx-1 and Ngn-3 efficiently induces liver-to-pancreas reprogramming of mature hepatocytes (34).

The present study represents a model for the study of the transition from hepatic to insulin-secreting cells. Beyond the scope of this present study, experiments to demonstrate long-term functionality of the transdifferentiated cells will be necessary to prove the therapeutic efficacy of the protocol used to generate beta-cells.

Although a number of pancreatic transcription factor genes involved in beta-cell maturation have already been elucidated, further research is needed in order to characterize fully all the factors required in the conversion from hepatic to insulin-producing cells. This characterization will lead to a better understanding of the cellular and molecular events taking place during nuclear reprogramming and, thus, to optimization of protocols for obtaining an efficient number of functional beta-cells for transplantation.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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