Rapid Publication

Insulin Production by Human Embryonic Stem Cells

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Type 1 diabetes generally results from autoimmune destruction of pancreatic islet β-cells, with consequent absolute insulin deficiency and complete dependence on exogenous insulin treatment. The relative paucity of donations for pancreas or islet allograft transplantation has prompted the search for alternative sources for β-cell replacement therapy. In the current study, we used pluripotent undifferentiated human embryonic stem (hES) cells as a model system for lineage-specific differentiation. Using hES cells in both adherent and suspension culture conditions, we observed spontaneous in vitro differentiation that included the generation of cells with characteristics of insulin-producing β-cells. Immunohistochemical staining for insulin was observed in a surprisingly high percentage of cells. Secretion of insulin into the medium was observed in a differentiation-dependent manner and was associated with the appearance of other β-cell markers. These findings validate the hES cell model system as a potential basis for enrichment of human β-cells or their precursors, as a possible future source for cell replacement therapy in diabetes. *Diabetes* 50:1691–1697, 2001

Approximately 5–10% of all diabetic individuals suffer from type 1 diabetes. Recent studies have emphasized the importance of strict glycemic control in order to reduce ophthalmologic, neurological, and renal complications of the disease (1). Yet, pancreatic cell and islet cell replacement is currently considered the only curative therapy. Indeed, this approach was recently shown to reverse glomerular lesions in patients with diabetic nephropathy (2). The promise of this approach has recently been further strengthened by a report of the use of an improved, less hazardous glucocorticoid-free immunosuppresive regimen in islet allograft transplantation (3). However, the shortage of donations is a primary obstacle preventing this therapeutic modality from becoming a practical solution. Thus, attention has focused on the use of alternative sources such as xenografts, which have other disadvantages, including the potential risk of undetermined zoonotic infections (4). It has also been reported that β-cell lines derived from rodents might provide an unlimited source for cell replacement therapy (5–7). In addition to the problem inherent in xenobiotic sources, such cell lines have been shown to display phenotypic instability, with loss of insulin biosynthesis and regulated secretion while proliferating. Another more recently described approach involves extending the β-cell phenotype to other tissues using in vivo gene transfer (8,9), either by expressing the insulin gene or an insulin gene analogue under the control of a glucose sensitive promoter or by ectopic expression of insulin promoter factor-1 (IPF1)/pancreatic and duodenal homeobox gene-1 (PDX1) (10).

The establishment of pluripotent human embryonic stem (hES) cells (11,12) and embryonic germ (EG) cells (13) have introduced a new potential source for cell therapy in type 1 diabetic patients, especially in light of recent successes in producing glucose-sensitive insulin-secreting cells from mouse embryonic stem (ES) cells (14). hES cells grow as homogeneous and undifferentiated colonies when they are propagated on a feeder layer of mouse embryonic fibroblasts (MEFs) (11). As previously shown, they have a normal karyotype and express telomerase and embryonic cell-surface markers. Removal from the MEF feeder layer is associated with differentiation into derivatives of the three EG layers, as evident from teratomas formed after subcutaneous injection in nude mice (11). Endodermal markers, but not insulin expression, were reported in a previous general survey of different growth conditions and differentiation markers in EG cells (15). Using reverse transcriptase-polymerase chain reaction (RT-PCR) applied to RNA extracted from differentiated hES cells, detection of a variety of differentiated cell markers, including insulin, was reported (16). However, quantitative aspects, including elaboration of insulin into the medium and percentage of insulin-producing cells, were not determined. Such information is crucial to assess the feasibility of using hES cells as a potential source for β-cell replacement therapy. These questions were addressed in the current study, using the differentiation of the H9 line of hES cells as described by Thomson et al. (11). Using a variety of experimental approaches, we found abundant cells with β-cell features, most notably including insulin production and secretion.

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bFGF, basic fibroblast growth factor; dNTP, deoxyribonucleotide; EB, embryoid body; EG, embryonic germ; ES, embryonic stem; GK, glucokinase; hES, human ES; hIns, human insulin; IPF1, insulin promoter factor-1; MEF, mouse embryonic fibroblast; Ngn3, neurogenin-3; Oct4, octamer-binding transcription factor-4; PDX1, pancreatic and duodenal homeobox gene-1; PCR, polymerase chain reaction; RT, reverse transcriptase.

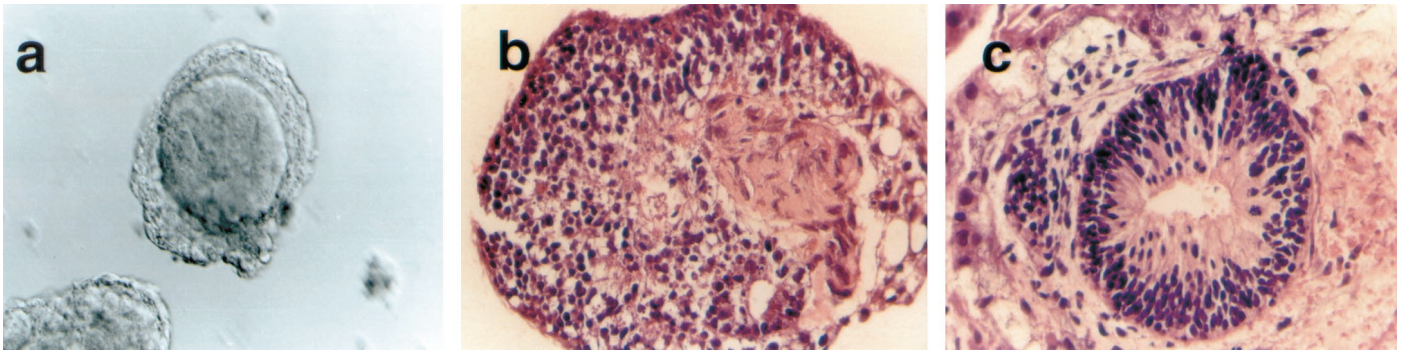

FIG. 1. Differentiation of hES cells in suspension culture. *A*: Simple EB 3 days after removal of MEF and growing in suspension culture. EBs were collected every 3 days, fixed in 10% neutral-buffered formalin, dehydrated in graduated alcohol, and embedded in paraffin. Then, 5-μm sections were stained with hematoxylin and eosin day 3 (*B*) and day 17 (*C*) after differentiation. Original magnification 20× (*A*) and 40× (*B* and *C*).

RESEARCH DESIGN AND METHODS

Tissue culture. Large stocks of primary MEFs were prepared as described by Robertson (17) and stored in liquid nitrogen. After each thaw, cells were used for only 3–5 passages.

The hES-H9 cells were maintained in the undifferentiated state by propagation in culture on a feeder layer of MEFs that had been mitotically inactivated by γ-irradiation with 35 Gy and plated on gelatin-coated six-well plates. Cells were grown in knockout Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 20% serum replacement (Gibco), 1% nonessential amino acids (Gibco), 0.1 mmol/l 2-mercaptoethanol (Gibco), 1 mmol/l glutamine (Biological Industries, Bet-Haemek, Israel), 4 ng/ml human recombinant basic fibroblast growth factor (bFGF) (PeproTech, Rocky Hill, NJ). Cultures were grown in 5% CO_2, 95% humidity, and were routinely passaged every 4–5 days after disaggregation with 0.1% collagenase IV (Gibco).

Induction of differentiation in hES cells. Methods for the induction of differentiation in mouse ES cells were applied herein for the induction of hES differentiation (17,18). In brief, ~10^7 undifferentiated hES cells were disaggregated and cultured in suspension in 100-mm bacterial-grade petri dishes (Greiner, Frickenhausen, Germany), which resulted in induction of synchronous differentiation characterized by initial formation of small aggregates and followed by the acquisition of the configuration of embryoid bodies (EBs) (19). Alternatively, hES colonies were left unpassaged until confluence (~10 days) and were replated on gelatinized six-well tissue culture plates in the absence of a feeder layer. The cells spontaneously differentiated to an array of cell phenotypes. The growth media used in differentiation are described above.

Histological analysis. EBs were collected at indicated intervals, washed three times with ice-cold phosphate-buffered saline, fixed overnight in 10% neutral-buffered formalin, dehydrated in graduated alcohol (70–100%), and embedded in paraffin. For general histomorphology, 5-μm sections were stained with hematoxylin/eosin.

Immunohistochemistry. Deparaffinized 5-μm sections were incubated for 90 min at room temperature with the primary antibody: polyclonal guinea pig anti-swine insulin, 1:100 dilution (Dako), followed by incubation with goat anti-rabbit biotinylated secondary antibody. Detection was accomplished using streptavidin-peroxidase conjugate and aminoethyl carbazole (or diaminobenzidine tetrahydrochloride) as a substrate (Histostain-SP kit; Zymed Lab). Counterstaining was carried out with hematoxylin. Nonimmune serum was used as a negative control, and normal human pancreas paraffin sections were used as positive controls.

Morphometric studies. To estimate the relative percentage of cells that stained positive by immunohisochemistry, morphometric measurements were conducted as previously described (20).

Insulin detection assay. Adherent cells, MEFs, undifferentiated hES cells, and cells that had differentiated spontaneously in vitro for >20 days were grown in six-well plates. Cells were washed three times with serum-free medium containing 25 mmol/l glucose and incubated in 3 ml of serum-free medium for 2 h. For suspended EBs, experiments were performed in 50-mm bacterial-grade petri dishes. A total of 60–70 EBs per dish were exposed for 2 h to 3 ml of serum-free medium containing either 5.5 or 25 mmol/l glucose. Subsequently, conditioned media were collected, and insulin levels were measured using a microparticle enzyme immunoassay (AXSYM system Insulin kit code B2D010; Abbott) that detects human insulin with no crossreactivaty to proinsulin or C-peptide.

RT-PCR. Total RNA was isolated from undifferentiated hES cells and from in vitro differentiated hES cells growing as EBs or as high-density cultures at various stages of differentiation.

cDNA was synthesized from 7 μg total RNA using Moloney murine leukemia virus RT (Promega) in 1× transcription buffer containing 0.5 μmol/l oligo $dT_{(12–18)}$ (Gibco) and 400 μmol/l deoxyribonucleotides (dNTPs). Aliquots of cDNA were diluted 1:5 for IPF1/PDX1, neurogenin 3 (Ngn3), octamer-binding transcription factor (Oct4), GLUT1, and GLUT2 or 1:2 for insulin- and islet-specific glucokinase (GK). Subsequent PCR was as follows: 2.5 μl cDNA (for IPF1/PDX1, Ngn3, and Oct4) or 5 μl cDNA (for others), 1× PCR buffer, 400 μmol/l dNTPs, 100 ng of each primer pair, and 1 U *Taq* polymerase. After initial hot start for 5 min, amplification continued with 28 cycles for β-actin, 31 cycles for GLUT1, 40 cycles for GLUT2, 38 cycles for GK, 36 cycles for insulin, 37 cycles for Oct4, and 35 cycles for IPF1/PDX1 and Ngn3. Denaturation steps were at 94°C for 1 min, annealing at 58, 52, 50, 67,62, 55, 52, and 60°C for 1 min, respectively, and extension was at 72°C for 1 min and a final polymerization for 10 min. The amplified products were separated on 1.5% agarose gels. Each PCR was performed in duplicate and under linear conditions. The forward and reverse primer sequences used for determination of human insulin (hIns), IPF1/PDX1, Ngn3, and β-actin were as follows: hIns: 5′-GCC TTT GTG AAC CAA CAC CTG-3′, 5′-GTT GCA GTA GTT CTC CAG CTG-3′ (261-bp fragment); IPF1: 5′-CCC ATG GAT GAA GTC TAC C-3′, 5′-GTC CTC CTC CTT TTT CCA C-3′ (262-bp fragment); Ngn3: 5′-CTC GAG GGT AGA AAG GAT GAC GCC TC-3′, 5′-ACG CGT GAA TGG GAT TAT GGG GTG GTG-3′ (948-bp fragment); and β-actin: 5′-CAT CGT GGG CCG CTC TAG GCA C-3′, 5′-CCG GCC AGC CAA GTC CAG GAC GG-3′ (508-bp fragment). The primer sequences used for determination of GLUT1, GLUT2, GK, and Oct4 were as previously described (21–23), the amplified fragments being 310, 398, 380, and 320 bps, respectively.

Statistics. Results are expressed as means ± SE, and comparisons were conducted using the unpaired Student's *t* test.

RESULTS

The H9 line of hES cells was used. These cells grow as homogeneous and undifferentiated colonies when they are propagated on a feeder layer of MEFs. Accordingly, spontaneous in vitro differentiation of H9 cells was investigated after removal of cells from the MEF feeder layer using two different model systems. Cells grown under adherent conditions in tissue culture plates, in the absence of MEFs, displayed a pleiotropic pattern with numerous morphologies, as previously described (24). In contrast, in vitro differentiation in suspension culture resulted in a more consistent pattern, with the formation of discrete EBs. One day after transfer to bacterial-grade petri dishes, cells failed to adhere and formed small aggregates. After 3 days under these conditions, EBs acquired a simple structure with primitive endodermal layers surrounding inner cells (Fig. 1*A*) and then continued to grow in size and developed a more cystic structure (400–700 μm in diameter). Subsequent studies were carried out using immunohistochemistry to determine the spatial and temporal pattern and to

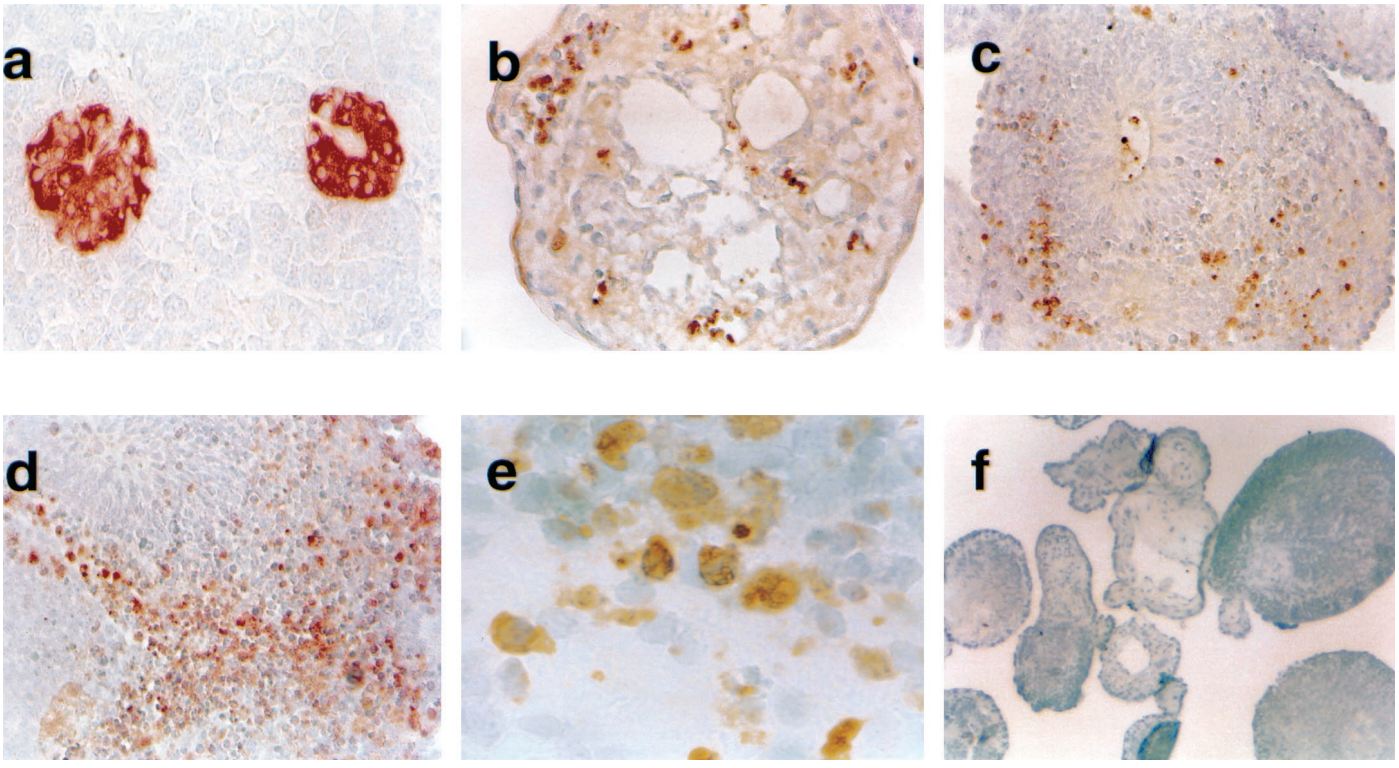

FIG. 2. Insulin-expressing cells in EBs. Immunohistochemistry was performed on paraffin sections as described in RESEARCH DESIGN AND METHODS. *A*: Normal human pancreas was used as a positive control (40× magnification). *B–D*: EBs at day 19 after differentiation (original magnification 40×). *E*: EB at 19 days after differentiation (100× magnification), where the plane of section shows cells in which the cytoplasmic localization of staining is evident. *F*: EBs at 19 days with nonimmune control serum (10× magnification).

obtain an estimate of the relative density of cells in suspension-cultured EBs with insulin-producing capability. Hematoxylin and eosin staining of paraffin-embedded sections was used to provide the overall histological morphology of EBs. Organization of EBs started as early as day 3 after removal from MEFs and transfer to suspension culture. With progressive days in suspension culture, more complex structures became evident, such as epithelial- or endothelial-like cells lining hollow structures or cysts (Fig. 1*B* and *C*).

After EB development, these cells were collected every 3 days until day 19 for immunohistochemistry using anti-insulin antibody. Pancreatic islets were used as a positive control (Fig. 2*A*), and no staining was evident using nonimmune serum (Fig. 2*F*). Occasional cells expressing insulin were evident as early as 14 days of differentiation, with a progressive increase in number through day 19. Insulin-expressing cells were found either scattered throughout the EBs or organized into small clusters (Fig. 2*B–D*). Higher magnification confirmed that immunohistochemical staining was cytoplasmic, as expected (Fig. 2*E*). Among EBs, which stained positively for insulin (60–70%), an average of 1–3% cells positively stained at maximum density. The remaining 30–40% of EBs were negative for insulin staining.

To characterize these insulin-containing cells, which are interspersed among the mixed population of spontaneously differentiating adherent hES, insulin elaborated into the medium was measured by enzyme immunoassay in undifferentiated hES, differentiated hES, and MEF cells. Growth medium contained serum replacement and 25 mmol/l glucose, which is essential for hES viability. Under each of the experimental conditions tested, immunoreactive insulin concentration was measured at the end of a 2-h incubation period in 3 ml of serum-free medium in either six-well plates (adherent cells) or 50-mm petri dishes (EBs). In adherent cells, insignificant immunoreactive insulin could be detected in media harvested from undifferentiated hES (5.6 ± 0.6 μU/ml, $n = 6$) (Fig. 3*A*), and none could be detected from a feeder layer without overlying hES. However, in media harvested after 22 and 31 days of differentiation, insulin concentrations were as follows: 126.2 ± 17.7 μU/ml ($n = 12$) and 315.9 ± 47 μU/ml ($n = 7$), respectively (Fig. 3*A*). Similarly, as shown in Fig. 3*B*, insulin release was significantly greater from 20- to 22-day EBs (60–70 EBs per dish), as compared with undifferentiated hES. No significant difference in insulin concentration was observed when incubations were carried out at a 5.5-mmol/l ambient medium glucose concentration (158 ± 16 μU/ml, $n = 6$) or at a 25-mmol/l ambient medium glucose concentration (146.2 ± 22.1 μU/ml, $n = 6$).

The foregoing results prompted us to examine the expression of other β-cell–related genes using RT-PCR analysis of total RNA extracted from undifferentiated and differentiated hES cells. As shown in Fig. 4, insulin mRNA was detected in differentiated cells but not in undifferentiated hES. In parallel, islet GK and GLUT2 genes were also identified after but not before differentiation. Similar results were obtained using either EBs or high-density adherent culture conditions. On the other hand, the GLUT1 isotype, a constitutive glucose transporter, was widely expressed in all forms of hES, as well as in human fibroblasts. Expression of three transcription factors was examined. As expected, mRNA expression of Oct4, a marker of the

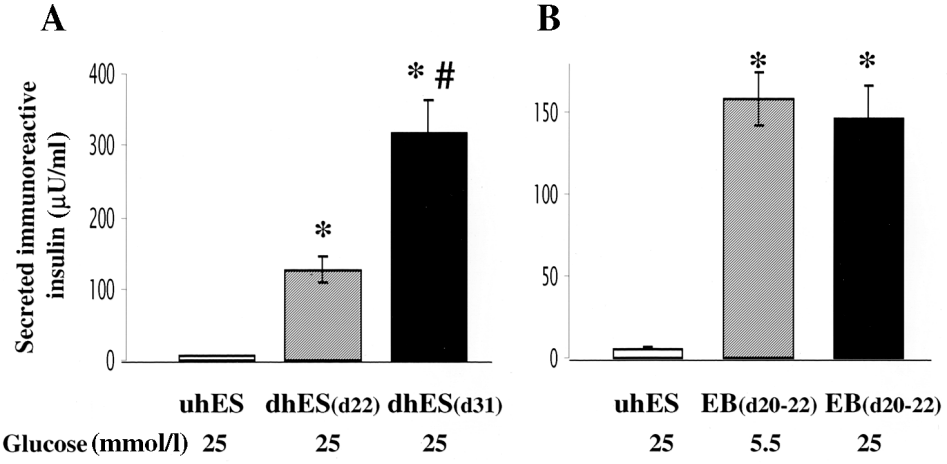

FIG. 3. Insulin secretion at various glucose concentrations and growth conditions. *A*: Undifferentiated hES (uhES) cells were cultured in knockout medium (n = 6) or were allowed to differentiate in high-density adherent conditions (dhES) for 22 (n = 12) and 31 days (n = 7). *B*: hES grown in suspension as EBs (60–70 EBs per dish) for 20–22 days (n = 6). Cultures were exposed to 3 ml of serum-free medium for 2 h containing either 25 or 5.5 mmol/l glucose, as indicated. The media were harvested after incubation, and insulin concentration was measured using enzyme immunoassay as described in RESEARCH DESIGN AND METHODS. Data are means ± SE. *P < 0.0001 vs. uhES; #P = 0.0004 vs. dhES-d22.

pluripotent state (25,26), was detected in undifferentiated hES but decreased progressively during the subsequent 3 weeks of differentiation (Fig. 4*C*). We also demonstrated that differentiating hES cells express IPF1/PDX1 and Ngn3 transcription factors (Fig. 4*C*); together they have been shown to contribute to the regulation of pancreatic and endocrine cell differentiation (27–30).

DISCUSSION

As has been shown during the past two decades, embryonic development of the pancreas is the result of several distinct but interacting mechanisms involving growth factors, epithelial-mesynchymal interactions (31), and extracellular matrix that eventually regulate the expression of diverse transcription factors (27,32–33). However, the initial signal in the cascade of events that eventuate in the commitment of gut endoderm to develop into pancreatic tissue is still obscure. In our study, we provide evidence that a pathway for producing insulin-secreting cells with additional β-cell features is not an infrequent outcome in the course of spontaneous differentiation of hES cells in culture, under the conditions that we used. This observation is a prerequisite for experimental strategies based on the enrichment of spontaneously appearing β-cells or their precursors for cell replacement therapy. The cells described herein produce and secrete insulin and express two essential genes, GLUT2 and islet-specific GK, that are believed to play an important role in β-cell function and glucose-stimulated insulin secretion (34–36). The possibility that the insulin-staining cells are unrelated to β-cells and are of extraembryonic or other origin (37) is highly unlikely, in view of the other markers identified, including the temporal course of appearance of β-cell developmental markers, and in view of the secretion of fully processed insulin.

Although we have not demonstrated glucose responsiveness, we cannot conclude that the cells are glucose unresponsive. As long as β-cells are not in a homogeneous or enriched state, rather presenting among other cell types, we cannot isolate the effects of glucose from countervening effects of other secretagogues. Furthermore, in the absence of homogeneous cell populations, comparisons based on different experimental conditions are not readily quantified because of the heterogeneity among EBs and the difficulty in normalizing insulin response to parameters such as protein or DNA content (38). The fine-tuning of insulin secretion in response to glucose requires cross-talk between adjacent β-cells caused by functional heterogeneity, and it has been shown that isolated β-cells function differently compared with β-cells found in clusters or pseudoislets (39,40). Moreover, long-term exposure to high glucose levels might affect the function of such insulin-producing cells and reduce their responsiveness to acute glucose changes, as has been previously reported in other systems (38). This high-glucose medium is needed to maintain the viable growth of hES in culture, but this does not rule out the possibility that protocols allowing growth of cells with insulin-producing capability in media containing lower glucose concentrations may restore glucose responsiveness. In any case, for treatment of diabetes with β-cell grafts derived from differentiated hES, it will be necessary to demonstrate stimulus-secretion coupling after obtaining enriched or homogeneous β-cell cultures, as has been demonstrated for mouse ES-derived β-cells (14).

Recently, islet cells were successfully generated in vitro from pancreatic stem cells of humans and adult mice (41,42). However, the major practical limitation of this approach is the restricted number of cells that can be cultivated from human pancreases. Hence, hES cells may represent a reasonable potential alternative.

Nevertheless, before proceeding with additional efforts to establish this system as an alternative resource, several issues need to be clarified. For instance, in contrast to the results of in vitro differentiation of hES-1 and -2 lines reported by Reubinoff et al. (12), our H9 suspension cultures did not induce prominent cell death and did result in EB formation. This observation may indicate different characteristics of each hES-derived cell line. Currently used hES cells are not of clonal origin, despite their homogenous appearance in the undifferentiated state, suggesting the need to examine the in vitro differentiation of each hES-derived cell line independently or the need to

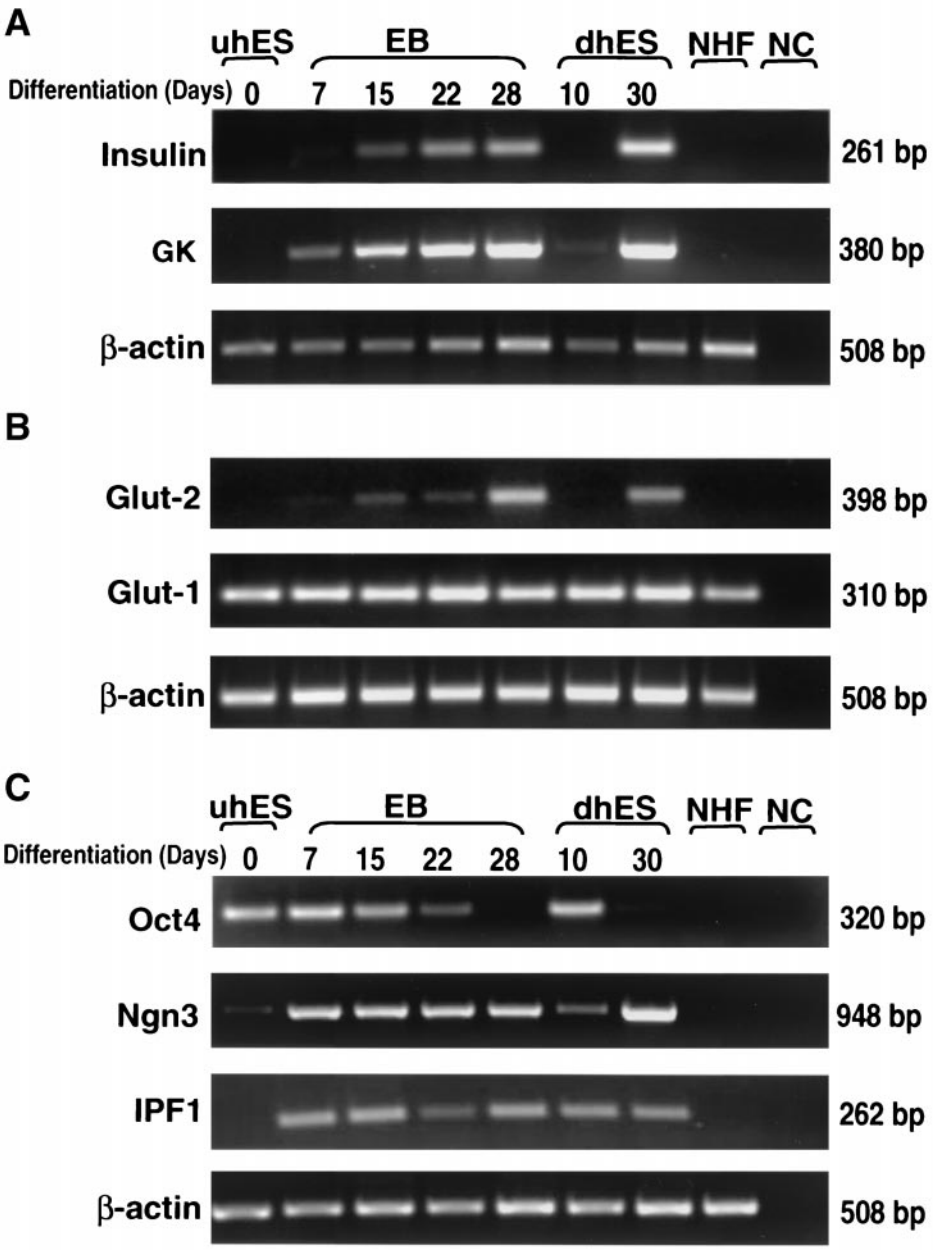

FIG. 4. Expression of β-cell–related genes. Total RNA was extracted from undifferentiated hES (uhES) cells, from differentiated hES growing either as EBs or as high-density adherent cell cultures (dhES) at various stages of differentiation, and from normal human fibroblasts (NHF). cDNA was synthesized from 7 μg total RNA and oligo dT primer. Aliquots of cDNA were diluted 1:2 for insulin and islet-specific GK or 1:5 for GLUT1, GLUT2, Oct4, Ngn3, and IPF1/PDX1 before PCR. β-actin served as an internal standard. NC indicates no cDNA. For insulin, GK, GLUT2, GLUT1, Oct4, Ngn3, IPF1/PDX1, and β-actin, 36, 38, 40, 31, 37, 35, 35, and 28 cycles were applied, respectively, for insulin, GK, and β-actin (*A*); for GLUT1, GLUT2, and β-actin (*B*); and Oct4, Ngn3, IPF1/PDX1, and β-actin (*C*).

examine clonal hES cell lines with well-defined differentiated responses to growth factors (43).

In terms of strategies for enriching the population of insulin-secreting hES-derived cell lines for further characterization and study, our results certainly indicate that the approach first described by Klug et al. (44) to enrich cardiomyocytes from mouse ES cells is potentially applicable. In the case of β-cells, the insulin promoter fused to a downstream selection marker could serve as the relevant selection tool. Indeed, this strategy was recently extended to enrichment of β-cells from mouse ES cells (14). Our findings indicate that even under conditions of spontaneous differentiation from nonclonal pluripotent hES cells, EBs are produced with a surprisingly high representation of cells with insulin-producing capacity, as compared with previous reports using mouse ES (6,14). Although this appears to occur spontaneously, it should be noted that the differentiation medium was supplemented with bFGF. Recently, Hart et al. (45) have suggested that FGF signaling may be involved in β-cell maturation, terminal differentiation, and post natal expansion. Tissue engineering estimates indicate that this is already a sufficient basis for enrichment protocols, based on the strategy described by Klug et al. (44). Certainly, additional protocols to enhance the starting number of β-cells in the mixed population of cells within the EBs can only improve the yield from enrichment strategies. Other limiting issues that may arise include possible senescence (46) of postdifferentiation

hES-cell derivatives attributed to loss of telomerase, as we have reported (24).

In conclusion, our findings clearly indicate that the complex differentiation pattern of hES cells includes a subset of cells that have many characteristics of β-cell function, including proinsulin and/or insulin production and insulin release, as well as the expression of other β-cell markers. Although not surprising, in view of the capacity of ES cells to differentiate into multiple different cell types, this finding is a necessary prerequisite for therapeutic strategies based on cell enrichment from hES cells as a source of cell replacement in type 1 diabetes.

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