# Artifactual Insulin Release From Differentiated Embryonic Stem Cells

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**Several recent reports claim the generation of insulin-producing cells from embryonic stem cells via the differentiation of progenitors that express nestin. Here, we investigate further the properties of these insulin-containing cells. We find that although differentiated cells contain immunoreactive insulin, they do not contain proinsulin-derived C-peptide. Furthermore, we find variable insulin release from these cells upon glucose addition, but C-peptide release is never detected. In addition, many of the insulin-immunoreactive cells are undergoing apoptosis or necrosis. We further show that cells cultured in the presence of a phosphoinositide 3-kinase inhibitor, which previously was reported to facilitate the differentiation of insulin$^+$ cells, are not C-peptide immunoreactive but take up fluorescein isothiocyanate–labeled insulin from the culture medium. Together, these data suggest that nestin$^+$ progenitor cells give rise to a population of cells that contain insulin, not as a result of biosynthesis but from the uptake of exogenous insulin. We conclude that C-peptide biosynthesis and secretion should be demonstrated to claim insulin production from embryonic stem cell progeny. *Diabetes* 53:2603–2609, 2004**

Recent progress has made islet transplantation from organ donors a promising therapy for patients with severe type 1 diabetes (1). As with all transplantation therapies, this treatment is hampered by a lack of suitable donors. This has focused interest on the potential use of embryonic stem (ES) cells to derive insulin-producing cells. The derivation of pancreatic cells from ES cells would also provide a tool to study pancreatic development and function.

ES cells are pluripotent cells derived from the inner cell mass of the blastocyst. These cells can be cultured indefinitely in an undifferentiated state but upon stimulation can differentiate to various cell types (2). Recent reports describe the derivation of insulin-containing cells from ES cells using different strategies (3–11). The rationale for several of these studies is the hypothesis that the pancreas and the central nervous system (CNS) share genetic and developmental pathways (12–14). Pancreatic endocrine cells share several characteristics with neurons (15), and insulin-producing cells have been observed in the invertebrate nervous system (16–18) and in primary cell cultures of mammalian fetal brain (19). Thus, many protocols for differentiation of ES cells were designed first to produce or select for neural progenitors defined by nestin expression (20) and then direct pancreatic islet differentiation in subsequent steps. Nestin is a filament protein expressed in neuroepithelial progenitor cells (21). Using minor modifications in the differentiation protocols, populations of nestin$^+$ ES cell derivatives have been expanded and differentiated to insulin-containing cells (5–9). However, one study has shown that the insulin contained in cells derived from nestin$^+$ progeny was due to uptake of exogenous insulin by apoptotic cells, rather than de novo insulin synthesis (22).

In this study, we investigate further the previous reports that ES cell–derived neural progenitors differentiate in vitro to insulin-containing cells (7–9) using both human- and mouse-derived ES cells. We extend previous studies by preparing ES cell derivatives that express Sox2, an SRY-related transcription factor (23) expressed in neuroepithelial progenitors (24), and then test whether Sox2$^+$ cells differentiate into insulin$^+$ cells that form the islet-like structures previously described (6,8). Furthermore, we address the effect of phosphoinositide 3-kinase (PI3K) inhibitors, which have been reported to drive the differentiation of ES cell–derived nestin$^+$ progenitors to insulin-containing cells (6). We find that nestin$^+$ progenitors give rise to a population of cells that release insulin when glucose is added to the media, but, notably, C-peptide release is never detected. The insulin release is variable and does not follow the anticipated release kinetics found in β-cells, suggesting that the observed insulin release cannot be viewed as authentic glucose-stimulated insulin secretion. The absence of C-peptide release suggests that

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bFGF, basic fibroblast growth factor; CNS, central nervous system; EB, embryoid body; ELISA, enzyme-linked immunosorbent assay; ES, embryonic stem; FITC, fluorescein isothiocyanate; Pdx1, pancreas duodenum homeobox-1; PI3K, phosphoinositide 3-kinase; RIA, radioimmunoassay; TUNEL, transferase-mediated dUTP nick-end labeling.

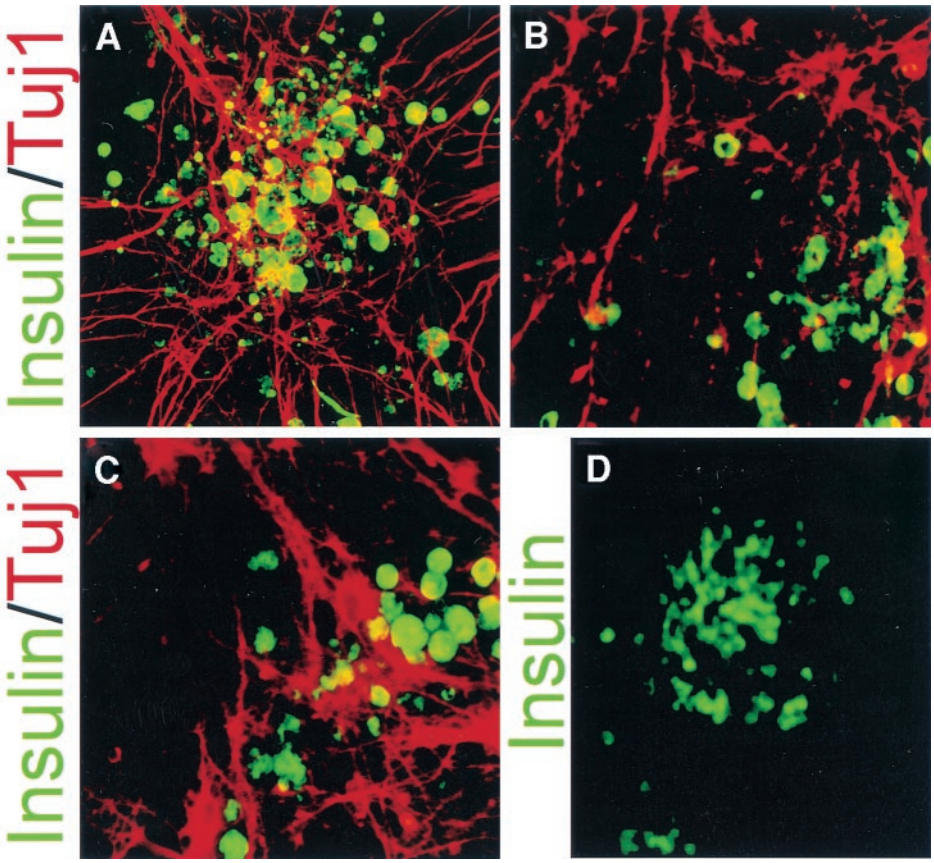


**FIG. 1. Characterization of differentiated ES cells. Insulin$^+$ cells develop in close association with neurons (*A–C*). Human ES cells (*A*) and mouse ES cells (OS25; *B*) that were cultured according to protocol 1 as well as OS25 cells that were cultured according to protocol 2 (*C*) give rise to insulin$^+$ cells that are closely associated with Tuj1$^+$ neurons. Mouse ES cells that were cultured according to protocol 3 give rise to insulin$^+$ cells as previously reported (*D*).**

these cells do not produce insulin. We suggest that C-peptide secretion should be measured in addition to insulin in future studies, preferably in combination with other assays for de novo synthesis of insulin, before claiming the generation of insulin-producing cells by differentiation of ES cells.

## RESEARCH DESIGN AND METHODS

**Cell culturing.** Mouse ES cells, OS25, where the selection marker/reporter gene β *geo* has been inserted into the S*ox2* locus (23), were cultured as previously described (8) with the following minor modifications (protocol 1): embryoid bodies (EBs) were grown for 5 days in suspension culture. Precursor cells (stage 4) were expanded for 6 days on polyornithine (Sigma, St. Louis, MO)/laminin (Becton Dickinson Bioscience, Bedford, MA)-coated tissue culture plates or poly-D-lysine/laminin-coated coverslips (BIOCOAT; Becton Dickinson Bioscience) in Dulbecco's modified Eagle's medium/F12 medium supplemented with N2, B27, and 10–20 ng/ml basic fibroblast growth factor (bFGF; all from Gibco/BRL, Gaithersburg, MD). Differentiated (stage 5) cells used for microarray analysis were cultured in 1 mmol/l nicotinamide (Sigma). EBs (stage 2) that were destined to be selected for expression of Sox2 (protocol 2) were cultured in media supplemented with $10^{-6}$ mol all-trans retinoic acid for the last 2.5 days. Selection of Sox2 expression in stage 3 was carried out in Dulbecco's modified Eagle's medium/F12 medium supplemented with N2, B27 (20), 20 ng/ml bFGF, and 200 μg/ml Geneticin (G418; all from Gibco/BRL).

Human ES cells (lines Sahlgrenska [SA] 001, SA002, and SA094 [25]) were cultured as described for mouse ES cells (8) with minor modifications (protocol 1). Briefly, undifferentiated ES cells were grown on mitomycin C–inactivated mouse embryonic fibroblast cells. EBs were generated by incubating ES cells in the absence of bFGF and leukemia inhibitory factor in suspension cultures for 4–9 days. EBs were subsequently plated on Cell+ tissue culture surface (Sarstedt, Nümbrecht, Germany) and glass coverslips in media with 20 ng/ml bFGF for 6–9 days. For selecting for nestin$^+$ precursor cells, medium was changed to serum-free ITSFn medium. Cells were trypsinized and plated on tissue culture dishes or glass coverslips precoated with poly-L-ornithine (Sigma)/laminin (Gibco/BRL). For expanding the progenitors, cells were incubated in N2 serum-free medium for 5–7 days. For inducing differentiation of insulin-containing cell clusters, cells were incubated in N2 serum-free medium, without bFGF, and supplemented with 10 mmol/l nicotinamide (Sigma) for 5–14 days.

Four mouse-derived ES cell lines (JM1, Black6, Ins-lacZ, and pancreas duodenum homeobox-1 [Pdx1]-lacZ) were cultured as previously described by Hori et al. (6) (protocol 3), which is a protocol similar to the multistep protocol described by Lumelsky et al. (8) plus 10 μmol/l PI3K inhibitor (LY294002; Calbiochem, San Diego, CA) added during the last stage of differentiation (6). For some experiments, fluorescein isothiocyanate (FITC) insulin (Sigma) was substituted for regular insulin in the culture medium.

**Insulin and C-peptide release.** Differentiated mouse and human cells were washed in incubation buffer that contained Krebs-Ringer buffer with or without 24 mmol/l $NaHCO_3$ supplemented with 0.1 or 0.5% BSA. Cells were preincubated in incubation buffer with no or 3 mmol/l glucose for 30–60 min at 37°C. Differentiated mouse cells were incubated in 3, 5, 8, 11, or 20 mmol/l glucose in incubation buffer for 120 min at 37°C, whereas differentiated human cells were incubated in 5 or 20 mmol/l glucose in incubation buffer for 5 min. Supernatants were collected. Cells were lysed, and the total cellular protein content was determined by BioRad protein assay (BioRad, Richmond, CA) or BCA Protein Assay (Pierce, Rockford, IL). The pancreatic β-cell line INS-1E was used as positive control. At least three independent experiments were analyzed.

**Measurement of secreted insulin and C-peptide.** The amount of secreted insulin was determined by enzyme-linked immunosorbent assay (ELISA) as described elsewhere (26,27) using antibodies that recognize mouse and rat insulin or using a human insulin radioimmunoassay (RIA) kit (Pharmacia insulin RIA 100; Pharmacia & Upjohn Diagnostic, Peapack, NJ). Secreted mouse C-peptide was assayed using a RIA kit (Linco Research, St. Charles, MO) according to the manufacturer's instructions. Human C-peptide was analyzed using ELISA (Dako, Carpinteria, CA).

**Immunocytochemistry.** Cells were fixed in 1 or 4% paraformaldehyde in PBS. Immunocytochemistry was performed using standard protocols. Primary antibodies and dilutions were as follows: guinea pig anti-mouse insulin polyclonal antibodies, 1:1,000 (Novo Nordisk, Bagsvaerd, Denmark); guinea pig anti-human insulin polyclonal antibodies, 1:200 (Dako); guinea pig anti-human insulin polyclonal antibodies, 1:500 (Linco); rabbit anti-rat C-peptide I polyclonal antibodies, 1:2,000 (28); rabbit anti-rat C-peptide II polyclonal antibodies, 1:2,000 (28); mouse anti-human C-peptide monoclonal antibodies, 1:4,000 (29); guinea pig anti-human C-peptide polyclonal antibodies, 1:250

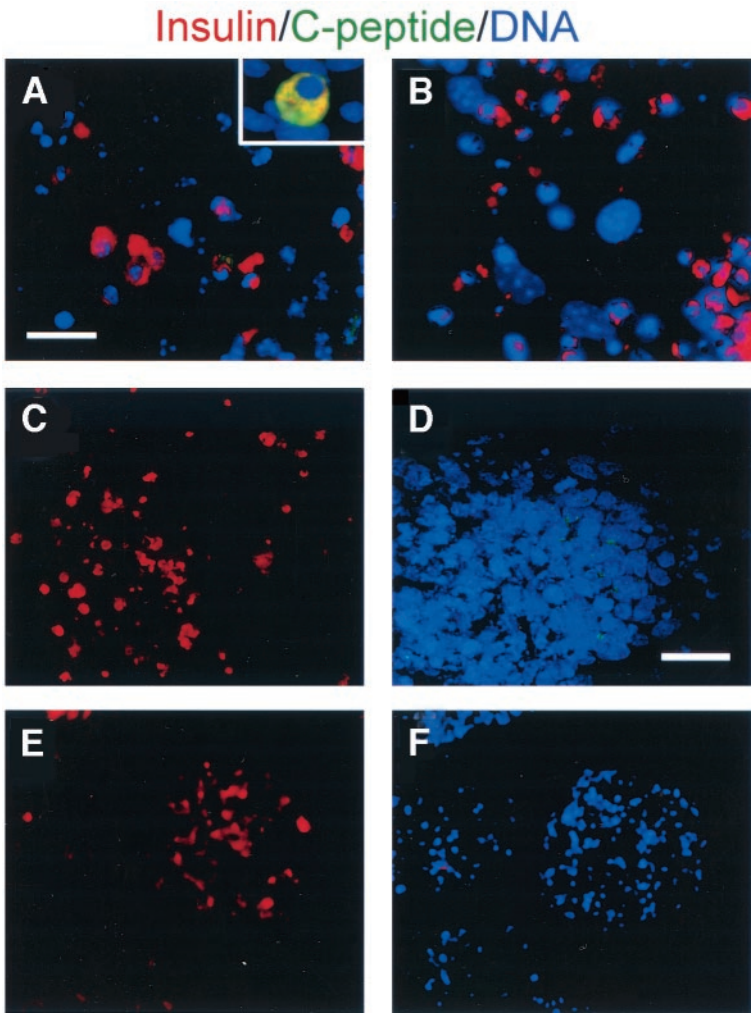


**FIG. 2. Lack of C-peptide expression in cells that were immunoreactive for insulin. Immunofluorescence staining for insulin and C-peptide. Insulin but not C-peptide is detected in differentiated cells. *A* and *B*: Differentiated mouse ES cells (OS25) that were cultured according to protocol 1 (*A*) and cells that were cultured according to protocol 2 (*B*). MIN6 cells, in which a clear colocalization of insulin and C-peptide is observed, were used as positive control (*A*, *insert*). *C* and *D*: Human ES cells that were cultured according to protocol 1. Green signal in *D* is background. *E* and *F*: Mouse-derived ES cells that were cultured according to protocol 3. Scale bars: 20 μm in *A*; 30 μm in *D*.**

(Linco); rabbit anti-glucagon polyclonal antibodies, 1:50 (Zymed, South San Francisco, CA); rabbit anti-rat nkx6.1 polyclonal antibodies, 1:4,000 (30); rabbit anti-Pdx1 polyclonal antibodies, 1:2,000 (gift from Dr. C. Wright, Vanderbilt University, Nashville, TN); mouse anti-rat β-III tubulin monoclonal antibodies, 1:50 (Sigma); rabbit anti-rat β-III tubulin polyclonal antibodies, 1:3,000 (Convance Research Products, Princeton, NJ); mouse anti-rat nestin monoclonal antibodies, 1:50 (Developmental Studies Hybridoma Bank, Iowa City, IA); and rabbit anti-human cleaved caspase-3 polyclonal antibodies, 1:50 (Cell Signaling Technology, Beverly, MA). Fluorescence-labeled secondary antibodies were used according to the manufacturer's suggestions (Jackson ImmunoResearch Laboratories, West Grove, PA) or tyramide signal amplification was applied according to the manufacturer's instructions (PerkinElmer Life Science, Wellesley, MA). Cell nuclei were visualized using DAPI (ICN Biomedicals, Cleveland, OH) or TO-PRO-3 (Molecular Probes, Eugene, OR). Nestin$^+$ cells were quantified after selection. Stage 3 cells were fixed and stained using nestin antibodies as previously described, and the number of nestin$^+$ cells was scored in five randomly picked areas. The mean percentage ± SD is presented. Immunofluorescent stainings were examined using confocal laser scanning microscopy (Figs. 1*A*, 2*C* and *D*, and 3*C* and *D*) or epifluorescent microscopy.

**Transferase-mediated dUTP nick-end labeling assay.** Apoptosis in differentiated (stage 5) cells was analyzed by fluorescent transferase-mediated dUTP nick-end labeling (TUNEL) assay (ApopTag PLUS Fluorescein In Situ Apoptosis Detection kit; Serologicals, Norcross, GA; or Fluorescent Apoptosis Detection kit; Promega, Madison, WI). Cells were fixed in 1 or 4% paraformaldehyde in PBS and processed according to the manufacturer's instructions or as previously described (22).

**Construction of 4K murine array.** The 3,999 cDNA fragments of the present gene set were analyzed and subsequently purified using a 96-well PCR purification system (Qiagen, Valencia, CA) according to the manufacturer's recommendation, mixed with an equal volume of DMSO, and spotted in duplicate onto silanized glass slides (type7*; Amersham Pharmacia Biotech, Piscataway, NJ) using a Generation III microarray spotter (Amersham Pharmacia Biotech). Spotted DNA was cross-linked to slides by ultraviolet irradiation (50 mJ) in a ultraviolet cross-linker (Stratagene, La Jolla, CA).

**Microarray experiments.** OS25 mouse ES cells were cultured as described (8) with previously described modifications (protocol 1). RNA from EBs (stage 2) to fully differentiated cells (stage 5) was purified using SV Total RNA Isolation System (Promega) according to the manufacturer's instructions. Three different RNA isolations were made from each stage. Total RNA was reverse-transcribed in the presence of aminoallyl-modified nucleotides followed by a coupling of the aminoallyl groups to Cy5-ester (Amersham Biosciences) as previously described (31). The spotted slides were prehybridized in 2× SSPE + 0.2% SDS for 90 min and washed. Labeled probe corresponding to 17 pmol Cy5 dye was hybridized under coverslips to arrays in 1× Microarray Hybridization Buffer Version 2 (Amersham) that contained 1 mg poly-dA$_{(70)}$ and 50% formamide. Arrays were incubated for 16 h at 42°C in a HybChamber (GeneMachine [http://genome.nhgri.nih.gov/genemachine]). After hybridization, slides were immersed in 1× SSC + 0.2% SDS to remove coverslips and subsequently washed repeatedly in 1× SSC + 0.2% SDS at 55°C and room temperature, followed by a final wash in water. Slides were scanned in an Axon 4000B scanner (Axon Instruments, Burlingame, CA), and image analysis was done using SpotFinder (Amersham).

**Data analysis.** All data analysis for the microarray study was done using the R environment and Bioconductor packages (32). The spot intensities were corrected for background as described previously (33), and q-spline normalization (34) was used to make expression levels comparable. The normalized values were subjected to ANOVA analysis.

**Quantitative PCR.** Verification of results obtained by the microarray analysis was performed using quantitative PCR. Quantification of selected transcripts was done using the LightCycler and DNA Master SYBR Green I (Roche, Palo Alto, CA). SuperScriptII reverse transcriptase (Invitrogen, San Diego, CA) was used for synthesizing first-strand cDNA according to the manufacturer's instruction (primer sequences and PCR conditions are available on request). Serial dilutions of first-strand cDNA from the pancreatic cell line

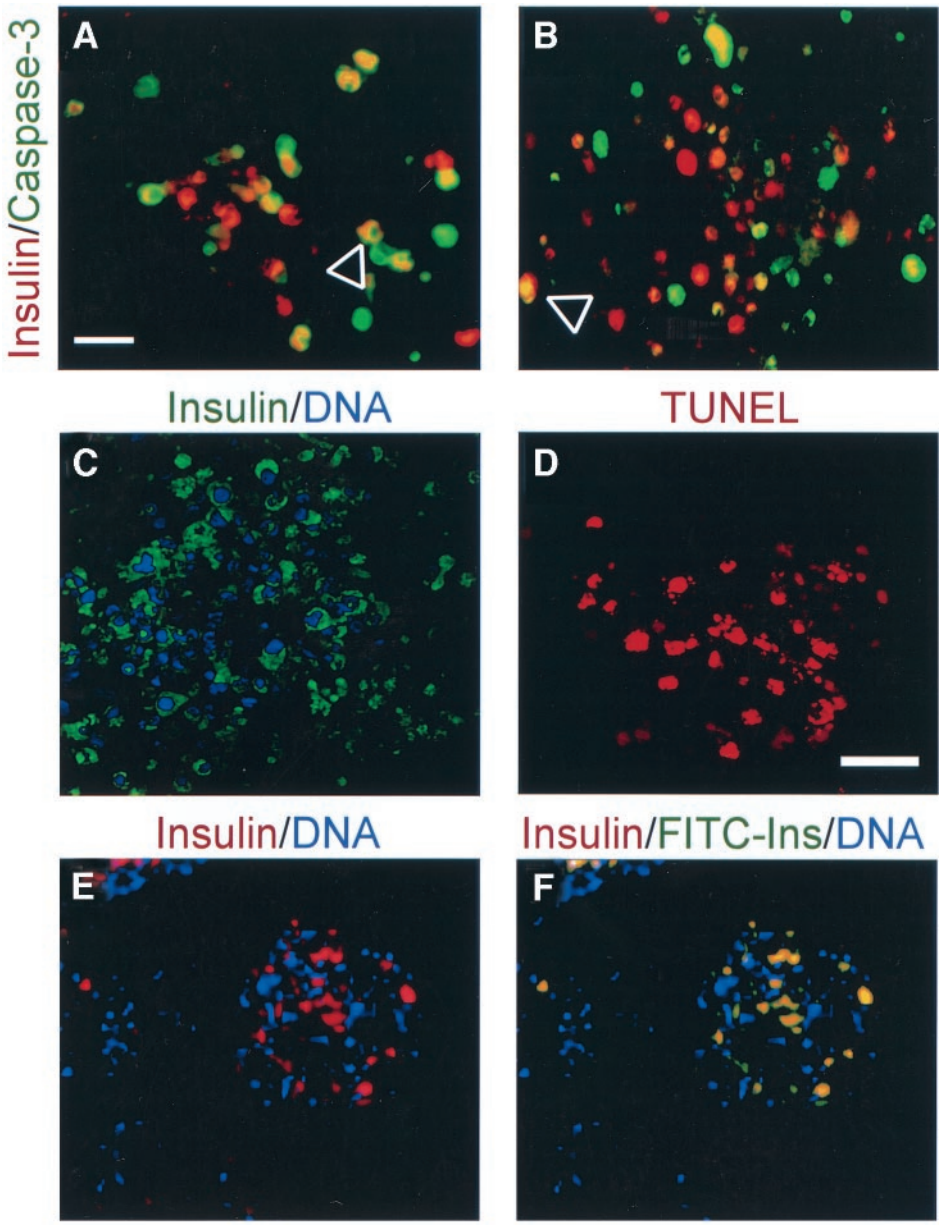


**FIG. 3. Insulin-immunoreactive cells are apoptotic and take up insulin from the culture media. Insulin$^+$ differentiated OS25 ES cells that were cultured according to the protocol 1 (*A*) and cells that were cultured according to protocol 2 (*B*) express the apoptotic marker cleaved caspase-3 (arrowheads). *C* and *D*: Insulin-containing cell clusters that were derived from human ES cells that were cultured according to protocol 1 have small, condensed nuclei and are TUNEL$^+$. *E* and *F*: Mouse-derived ES cells that were cultured according to protocol 3 take up FITC-labeled insulin when present in the culture media. Scale bars: 20 μm in *A*; 30 μm in *D*.**

βTC6 or cDNA mouse brain (Clontech, Palo Alto, CA) were used as standards in all experiments.

**X-gal staining.** Fixation of cells and β-galactosidase staining was performed as previously described (22).

## RESULTS

**Generation of insulin-containing cells from ES cells.** Several mouse and human ES cell lines were cultured according to different protocols that all were designed first to produce or select for neural progenitors, as defined by expression of nestin (21), and then direct islet differentiation in subsequent steps. Nestin$^+$ cells were obtained by culturing cells in serum-free media (protocol 1 [8]) and by culturing a mouse ES cell line expressing the bifunctional selection marker/reporter gene β *geo* from the *Sox2* locus (OS25 [23]) in G418 after retinoic acid treatment (protocol 2). Sox2 is expressed in the neuroepithelium during mammalian development (24). We hypothesized that ES cell progeny expressing Sox2 would provide a cell population enriched for nestin$^+$ cells for further differentiation to insulin-containing cells as described by Lumelsky et al. (8). OS25 cells that were cultured in G418-containing medium (protocol 2) gave rise to a cell population that contained more nestin$^+$ cells (82.8 ± 7.3%) compared with cells that were cultured in serum-free conditions (51.7 ± 24.5%), as described by Lumelsky et al. (protocol 1). These cells were expanded and differentiated using neurotrophic factors and nicotinamide (8). Human ES cells SA001, SA002, and SA094 (25) were cultured according to the protocol described by Lumelsky et al. (protocol 1 [8]).

In separate experiments, several mouse ES cell lines (JM1, Black6, Pdx1-lacZ, and Ins-lacZ) were cultured according to the protocol described by Hori et al. (6) wherein the PI3K inhibitor LY294002 was included during the last stage of differentiation (protocol 3).

The observations of previous studies (6,8) were confirmed as all protocols generated insulin$^+$ cells (Fig. 1).

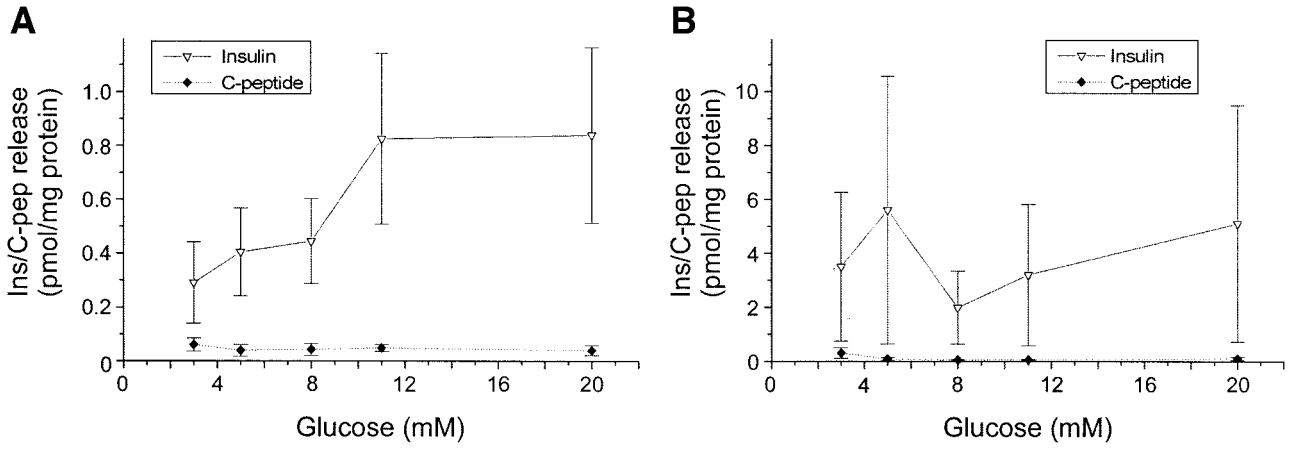


**FIG. 4. Glucose-stimulated insulin and C-peptide release of differentiated cells. Insulin (Ins) but not C-peptide (C-pep) is secreted from the cells after glucose stimulation. *A* and *B*: Mouse ES cells (OS25) that were cultured according to protocol 1 (*A*) or protocol 2 (*B*). Mean of four independent experiments ± SE presented.**

OS25 cells that were cultured according to protocol 1 and protocol 2 as well as human ES cells that were cultured according to protocol 1 gave rise to insulin$^+$ cells closely associated with neurons as previously reported (Fig. 1*A*–*C*). Insulin$^+$ cells typically displayed an unusual morphology, showing a large variation in cell size, but most were small and had condensed nuclei.

**Differentiated ES cell progeny release insulin but not C-peptide.** Glucose-stimulated insulin and C-peptide release from OS25 cells, cultured according to protocol 1 or protocol 2, were analyzed. Their functional response to physiological stimuli was compared with the pancreatic β-cell line INS-1E. Insulin and C-peptide release was determined by ELISA and RIA, respectively, after static incubation in buffer that contained 3, 5, 8, 11, or 20 mmol/l glucose. Cells that were cultured according to protocol 1 released 0.3–0.8 pmol insulin/mg total cellular protein (Fig. 4*A*), and cells that were cultured according to protocol 2 released even more insulin (2.0–5.6 pmol insulin/mg protein; Fig. 4*B*). However, the cells showed an abnormal glucose response compared with β-cells. In neither case did these cells release detectable amounts of C-peptide (Fig. 4), whereas both insulin and C-peptide secretion was readily detected in glucose-stimulated INS-1E cells (data not shown). Human ES cells that were cultured according to protocol 1 showed an abnormal glucose response, whereby maximal insulin release was seen already at 5 mmol/l glucose, with no further release at 20 mmol/l. Furthermore, no detectable amount of C-peptide was released from these cells (Table 1).

**Characterization of insulin-containing cells derived from nestin$^+$ precursors.** OS25 cells that were cultured according to protocol 1 or protocol 2, human ES cells that were cultured according to protocol 1, and mouse ES cells that were cultured according to protocol 3 were analyzed for insulin and C-peptide expression by immunocytochemistry. Insulin but not C-peptide immunoreactivity was detected in differentiated cells (Fig. 2). Control staining of the pancreatic β-cell line MIN6 showed a clear colocalization of insulin and C-peptide (Fig. 2*A*, *insert*). Insulin$^+$ cells also lacked the characteristic granular distribution of insulin found in β-cells. Insulin$^+$ cells derived from human ES cells according to protocol 1 were further characterized. No Pdx1 or glucagon expression could be detected in these cells by immunocytochemistry, whereas the transcription factor nkx6.1 and somatostatin were expressed in a subpopulation of insulin$^+$ cell clusters (data not shown). Moreover, a time-course transcriptional profile of OS25 ES cells that were cultured according to protocol 1 was performed using a 4K microarray that contained several genes implicated in pancreatic development. The expression of selected transcripts was confirmed by real-time PCR. Insulin, Pdx1, and prohormone convertase 2 showed no expression at any stage (data not shown). Somatostatin and pancreatic polypeptide showed an increase in transcription levels from EB stage to fully differentiated cells ($P < 0.005$), which is consistent with neural differentiation.

Mouse-derived ES cells with *lacZ* insertion downstream of the endogenous *insulin2* (Ins-lacZ) or *Pdx1* (Pdx1-lacZ) promoter were cultured according to protocol 3. Differentiated cells did not show any lacZ activity above background in either of the two cell lines (data not shown).

TABLE 1
Glucose-stimulated insulin and C-peptide secretion by human ES cells that were cultured according to protocol 1

| | Experiment 1 | | Experiment 2 | | Experiment 3 | | Total | |
|---|---|---|---|---|---|---|---|---|
| | Insulin | C-peptide | Insulin | C-peptide | Insulin | C-peptide | Insulin | C-peptide |
| Control | 1.4 ± 1.4 | ND | 0.2 ± 0.3 | ND | 0.2 ± 0.2 | ND | 0.5 ± 0.9 | ND |
| Glucose 5 mmol/l | 16.1 ± 6.3 | ND | 5.8 ± 1.8 | ND | 12.6 ± 4.0 | ND | 11.4 ± 6.1 | ND |
| Glucose 20 mmol/l | 8.4 ± 3.6 | ND | 6.3 ± 2.1 | ND | 7.2 ± 1.7 | ND | 7.3 ± 2.5 | ND |

Data are average concentration ± SD of insulin/total protein (ng/mg) in each of three experiments. Amounts of insulin secreted by cells that were cultured in glucose-free media (control) or in the presence of 5 and 20 mmol/l glucose. ND, not detected.

TABLE 2
Percentage of apoptotic or necrotic OS25 cells after differentiation according to protocols 1 and 2

| Culture conditions | Apoptotic/necrotic cells (%) |
|---|---|
| Protocol 1 | 41.9 ± 5.0 (1,043) |
| Protocol 2 | 63.7 ± 6.3 (1,749) |

Data are means ± SD (number of cells scored). Percentage of differentiated cells with condensed nuclei that were cultured according to the protocol described by Lumelaky et al. (protocol 1) or cells that were previously selected for Sox2 expression (protocol 2).

**Insulin-containing cells are apoptotic or necrotic.** Insulin$^+$ cells, independent of ES cell line and previous culture conditions, had small, condensed nuclei, indicating apoptosis or necrosis (Fig. 3*A*–*C*, *E*, and *F*). TUNEL assays as well as immunocytochemical staining for the apoptotic marker cleaved caspase-3 was performed to elucidate further the status of these cells. Insulin$^+$ cells derived from OS25 cells that were cultured according protocol 1 or protocol 2 expressed cleaved caspase-3 (Fig. 3*A* and *B*) and were TUNEL$^+$ (data not shown). Cells that were previously selected for Sox2 expression (protocol 2) had more cells with condensed nuclei (Table 2). Clusters of insulin$^+$ cells derived from human ES cells that were cultured according to protocol 1 showed a high incidence of apoptosis when analyzed by TUNEL assays (Fig. 3*C* and *D*). Several different mouse ES cell lines that were differentiated by protocol 3, which were cultured in the presence of FITC-conjugated insulin, concentrate FITC-insulin in the cells (Fig. 3*E* and *F*), indicating an uptake of insulin from the culture media. The use of LY294002 in protocol 3 caused massive cell death, and the remaining cells had small, condensed nuclei (Fig. 3*E* and *F*).

## DISCUSSION

Several studies have claimed the generation of insulin-producing cells from ES cells by expansion and differentiation of nestin$^+$ progenitor cells (5–9). Lumelsky et al. (8) reported that nestin$^+$ cells could differentiate to insulin-producing cells using neurotrophic factors and nicotinamide, and Hori et al. (6) claimed an improvement of this by adding a PI3K inhibitor, LY294002. Here, we repeated and further investigated these reports by using both human- and mouse-derived ES cells. These cells were cultured according to the protocol described by Lumelsky et al. (protocol 1) as well as using a PI3K inhibitor as described by Hori et al. (protocol 3). Furthermore, we investigated the potential of Sox2-expressing progenitors to generate insulin-containing cells (protocol 2) comparable with those derived by Lumelsky et al. (8).

For every cell line used, human and mouse, and for all differentiation protocols, we observed the formation of cell clusters that contained insulin$^+$ cells as previously described (6–9). We showed that these cells release insulin when glucose is added to the media, but C-peptide release is not detected. The amount of insulin released by mouse and human cells that were cultured according to protocol 1 (5–16 ng insulin/mg protein) is comparable with the amounts reported by Lumelsky et al. (8). The secretion of one adult mouse islet under similar conditions is ~3 ng (35). Because one islet contains ~0.5 μg of protein, this corresponds to 6 μg insulin/mg protein, or roughly a 400- to 1200-fold higher release from islets than from the insulin-containing ES derivatives. Furthermore, the secretory response to different glucose concentrations does not reflect the normal glucose-dependent insulin release observed in β-cells.

The lack of C-peptide secretion indicates that the released insulin is not the result of insulin biosynthesis by the cells, and the absence of C-peptide immunoreactivity is consistent with the contention that these cells do not make insulin. Furthermore, the absence of lacZ staining in Ins-lacZ ES cells that were differentiated according to protocol 3 is inconsistent with the contention that these cells transcribe the insulin gene. Our results contradict several recent publications (6–9). These discrepancies may be due to differences in staining conditions, as well as interpretation of immunocytochemical data, e.g., to conclude that cells are Pdx1 immunoreactive requires nuclear localization of the staining as well as proper positive and negative controls.

The similarities in gene expression patterns observed in the developing pancreas and CNS can confound conclusions about the differentiation of bona fide β-cells from ES cells when the only assay is the expression of gene markers. For example, somatostatin, pancreatic polypeptide, nkx6.1, and Glut-2 all have been detected in differentiated cells by RT-PCR, microarray analysis, or immunocytochemistry, from which it was concluded that β-like cells have been formed. Although these genes are indeed expressed in pancreatic endocrine cells, they are also expressed in the CNS (36–39).

Many insulin$^+$ cells contained small, condensed nuclei, suggesting apoptosis or necrosis. TUNEL assays and staining for the apoptotic marker cleaved caspase-3 confirmed a high incidence of apoptosis in insulin-containing cells. The lack of evidence for endogenous insulin biosynthesis and the high degree of cells undergoing apoptosis point to the conclusion that insulin release and insulin immunoreactivity can be explained by uptake of exogenous insulin that is present in the culture media. This is supported by the uptake of FITC-labeled insulin by differentiated cells. Moreover, Sox2-selected cells (protocol 2), which have more nestin$^+$ cells compared with cells that are cultured in serum-free medium (protocol 1), release more insulin after differentiation and have more apoptotic and necrotic cells, suggesting a correlation between insulin entrapment and apoptosis/necrosis. This may also be the case for cells that are cultured in the presence of PI3K inhibitor, where apoptosis is known to be enhanced (40). The nonphysiological release of trapped insulin from apoptotic and necrotic cells could explain the claims of partial rescue of mice with diabetes previously reported (5,6,8).

In conclusion, nestin$^+$ progenitors give rise to a cell population that releases insulin when glucose is added to the medium. The large variation in insulin release together with abnormal release kinetics and absence of C-peptide release suggest that the insulin found in these cells is not due to insulin biosynthesis. The large population of apoptotic cells and the finding that FITC-labeled insulin is concentrated in these cells indicate that exogenous insulin is trapped in apoptotic cells. However, we cannot rule out the possibility that a subpopulation of living insulin$^+$ cells adsorb insulin and secrete it back to the media when

stimulated by glucose or apparently appropriate pharmacological stimuli (8). In light of these findings, we suggest that C-peptide (in addition to insulin) secretion should be used in future studies, preferably in combination with other assays for de novo synthesis of insulin, to support conclusions that β-like cells have been produced by in vitro differentiation protocols.

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