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A New Experimental Protocol for Preferential Differentiation of Mouse Embryonic Stem Cells Into Insulin-Producing Cells

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Mouse embryonic stem (ES) cells have the potential to differentiate into insulin-producing cells, but efficient protocols for in vitro differentiation have not been established. Here we have developed a new optimized four-stage differentiation protocol and compared this with an established reference protocol. The new protocol minimized differentiation towards neuronal progeny, resulting in a population of insulin-producing cells with β-cell characteristics but lacking neuronal features. The yield of glucagon and somatostatin cells was negligible. Crucial for this improved yield was the removal of a nestin selection step as well as removal of culture supplements that promote differentiation towards the neuronal lineage. Supplementation of the differentiation medium with insulin and fetal calf serum was beneficial for differentiation towards monohormonal insulin-positive cells. After implantation into diabetic mice these insulin-producing cells produced a time-dependent improvement of the diabetic metabolic state, in contrast to cells differentiated according to the reference protocol. Using a spinner culture instead of an adherent culture of ES cells prevented the differentiation towards insulin-producing cells. Thus, prevention of cell attachment in a spinner culture represents a means to keep ES cells in an undifferentiated state and to inhibit differentiation. In conclusion, this study describes a new optimized four-stage protocol for differentiating ES cells to insulin-producing cells with minimal neuronal cell formation.

Key words: Embryonic stem cells; Differentiation; Insulin; Diabetes

INTRODUCTION

Embryonic stem cells are pluripotent cells purified from the inner cell mass of the blastocyst-stage embryo (11). They can be permanently cultured and represent an unlimited source of cells with the potential to differentiate into any kind of adult tissue. Because these cells possess the potential for cell replacement therapy they could substitute for the shortage of pancreatic islets required for implantation therapy of type 1 diabetes mellitus. Indeed, the generation of insulin-producing surrogate cells from ES cells with characteristics comparable to those of natural pancreatic β-cells has been attempted from mouse (16,26) and human embryonic stem cells (1,6). However, controversial results have emerged.

The differentiation protocol by Lumelsky et al. (16), originally developed from a culture protocol for neuronal differentiation (15), also has been used for differentiation towards insulin-producing cells. This protocol has been modified by several groups, but with limited success and divergent results (2,3,14,17). Cells differentiated according to the Lumelsky protocol are prone to cell death and may take up insulin from the differentiation medium, which is supplemented with very high concentrations of insulin (13,21,23). The neuronal orientation of the protocol has cast doubt upon its suitability for differentiation into insulin-producing cells, because the cell type obtained is neuronal with very limited constitutive insulin gene expression, low insulin content, and poor insulin release compared with a true insulin-secreting cell type (25).

Thus, the aim of this study was to develop an improved differentiation protocol suitable for generation of insulin-producing surrogate cells. We compared four protocols for ES cell differentiation towards insulin-producing cells. We reproduced the five-stage protocol by Lumelsky et al. (16) and compared this with a slightly modified protocol, in which fetal calf serum (FCS) was added to the final cultivation step of the differentiation procedure. We also designed a new optimized four-stage

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differentiation protocol. This protocol relies on the development of two-layered spherical clusters (embryoid bodies) from single cells in suspension, and on the outgrowth of these embryoid bodies (EBs) in serum-free adherent cell culture. We supplemented the differentiation medium during the final phase with FCS and nicotinamide. Our new optimized four-stage differentiation protocol minimized neuronal differentiation and increased expression of β-cell characteristic genes. Through removal of the nestin selection step and through removal of cell culture supplements that would promote survival of cells undergoing differentiation towards the neuronal lineage, we were able to generate cells with a typical β-cell phenotype.

It has been reported that cultivation of ES cells in a histotypic spinner culture improves maturation and differentiation (3). We therefore also differentiated ES cells in a spinner culture using our optimized four-stage differentiation protocol in combination with a magnetic glass ball steering system. Interestingly, the results show that in contrast to previous findings, cells in spinner culture retained an embryonic phenotype and showed no signs of differentiation towards insulin-producing cells.

MATERIALS AND METHODS

Materials

DMEM and DMEM/F-12 tissue culture media, glutamine, nonessential amino acids, bFGF, and Pluronic F-68 were obtained from Invitrogen (Karlsruhe, Germany). Fetal calf serum (FCS) embryonic stem cell grade and gentamicin were purchased from PAA (Vienna, Austria) and leukemia inhibitory factor (LIF) from Chemicon (Temecula, CA, USA). Insulin, transferrin, sodium selenite, putrescine, and progesterone were from Sigma (St. Louis, MO, USA). All primer pairs, including random hexamer primers, were synthesized by MWG (Munich, Germany). The RevertAid™ H^-M-MuLV reverse transcriptase was from Fermentas (St. Leon-Rot, Germany). The Biotherm™ Taq-polymerase as well as the dNTPs were from Genecraft (Münster, Germany). SybrGreen I was from Biozym (Hess. Oldendorf, Germany) and the plastic ware for the real-time-PCR reaction was from Abgene (Hamburg, Germany). Ac-DEVD-AMC was obtained from Biosource International (Camarillo, CA, USA) and AMC from Merck (Darmstadt, Germany). The ultrasensitive insulin ELISA was purchased from Mercodia (Uppsala, Sweden). Unless otherwise mentioned chemicals of analytical grade were obtained from Sigma or Merck (Darmstadt, Germany).

Cell Lines and Culture Conditions

The mouse embryonic stem (ES) cell line ES-D3 (10), which allows feeder-free culturing as well as coculture with inactivated primary mouse embryonic fibroblasts (feeder layer), was purchased from the American Tissue Culture Collection (ATCC, Manassas, VA, USA). In order to maintain the cells in an embryonic state, they were cultured on a feeder layer of mouse embryonic fibroblasts in stem cell medium (DMEM) containing 25 mM glucose and supplemented with 15% (v/v) FCS, 2 mM L-glutamine, 100 μM nonessential amino acids, 0.1 mM β-mercaptoethanol, 50 μg/ml gentamicin, and 1000 U/ml LIF in a humidified atmosphere at 37°C and 5% CO_2. Medium was changed daily and the cells were transferred for two passages on gelatin-coated tissue culture dishes to remove the feeder layer.

For differentiation the cells were cultured either according to a reference protocol (16), to a modified reference protocol, to a new optimized four-stage differentiation protocol, or to the new optimized four-stage differentiation protocol conducted in a histotypic spinner culture (Fig. 1). Detailed information about the reference protocol can be found elsewhere (16). To modify the reference protocol, the final differentiation medium was supplemented with different concentrations of FCS (1–10%). For differentiation with the new optimized four-stage differentiation protocol the cells were trypsinized and counted with a hemocytometer. One million cells were transferred onto a bacterial culture dish in medium as described above but devoid of LIF. Cells were then grown for up to 5 days in suspension. During this time cells formed EBs, which were allowed to settle down on gelatin-coated dishes in serum-free DMEM/F-12 medium supplemented with 25 μg/ml insulin, 50 μg/ml transferrin, 30 nM sodium selenite, 20 nM progesterone, 100 μM putrescine, 2 mM L-glutamine, 100 μM nonessential amino acids, and 10 ng/ml bFGF for 14 days. Thereafter the cells were cultured for 7 days in DMEM/F-12 medium supplemented with 25 μg/ml insulin, 50 μg/ml transferrin, 30 nM sodium selenite, 20 nM progesterone, 100 μM putrescine, 5% FCS, 2 mM L-glutamine, 100 mM nonessential amino acids, and 10 mM nicotinamide.

For differentiation in a spinner culture embryonic stem cells were trypsinized and counted with a hemocytometer. Subsequently 2×10^7 cells were resuspended in 100 ml DMEM medium containing 25 mM glucose, 15% (v/v) FCS, 2 mM L-glutamine, 100 μM nonessential amino acids, 0.1 mM β-mercaptoethanol, and 50 μg/ml gentamicin. The cells were then transferred into 125-ml glass ball spinner flasks (Techne, Jahnsdorf, Germany) and put under constant rotation at about 25 revolutions per minute on a Biosystem 4 remote-controlled stirrer connected to a Variomag Biomodul 40B (H+P Labortechnik, Oberschleissheim, Germany). After 5 days of differentiation the medium was changed to serum-free DMEM/F-12 medium supplemented with 25 μg/ml

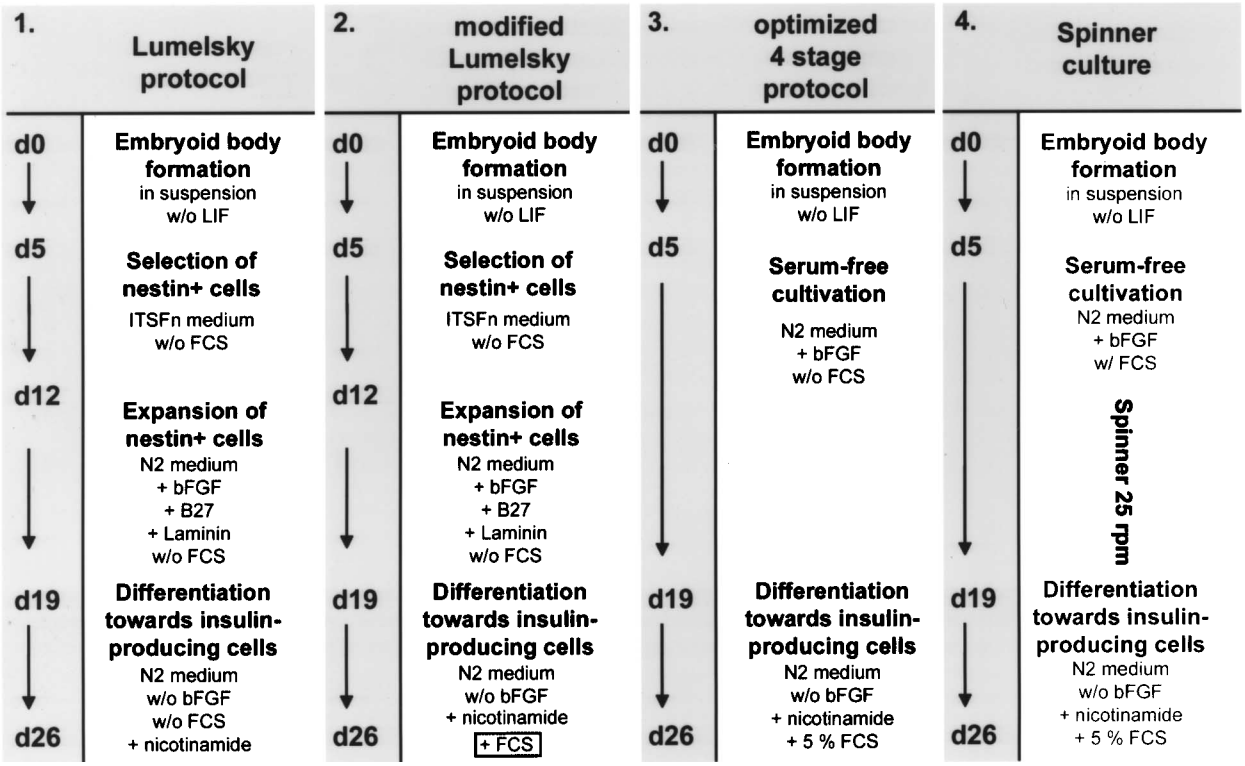

Figure 1. Schematic presentation of the four different culture protocols used for differentiation of mouse embryonic stem (ES) cells into insulin-producing cells.

insulin, 50 μg/ml transferrin, 30 nM sodium selenite, 20 nM progesterone, 100 μM putrescine, 2 mM L-glutamine, 100 μM nonessential amino acids, 10 ng/ml bFGF, and 0.1% Pluronic F-68 for 14 days. Thereafter the cells were cultured for 7 days in DMEM/F-12 medium supplemented with 25 μg/ml insulin, 50 μg/ml transferrin, 30 nM sodium selenite, 20 nM progesterone, 100 μM putrescine, 5% FCS, 2 mM L-glutamine, 1× nonessential amino acids, 0.1% Pluronic F-68, and 10 mM nicotinamide.

Molecular Biology

Total RNA was isolated from ES cells using the Chomczynski protocol (4). RNA was quantified photometrically and analyzed on a denaturating agarose gel. For cDNA synthesis random hexamers were used to prime the reaction of the RevertAid™ H⁻M-MuLV reverse transcriptase. The QuantiTect SYBR Green™ technology (Qiagen, Hilden, Germany), which uses a fluorescent dye that binds only double-stranded DNA, was employed. The reactions were performed using the DNA Engine Opticon™ Sequence Detection System (Biozym Diagnostik, Hess. Oldendorf, Germany). Samples were first denatured at 94°C for 3 min followed by 40 PCR cycles. Each cycle comprised a melting step at 94°C for 30 s, an annealing step at 62°C for 30 s, and an extension step at 72°C for 30 s. Primers used for qRT-PCR were designed exon-spanning to avoid amplification of genomic DNA (Table 1). All amplicons were in a size ranging from 100 to 300 base pairs. Each PCR amplification was performed in triplicate. The optimal parameters for the PCR reactions were empirically defined. The purity of the amplified PCR product was verified by melting curves. Data are expressed as relative gene expression after normalization to the β-actin housekeeping gene using the Qgene96 and LineRegPCR software (18,24).

Caspase 3 Enzyme Activity

The caspase 3 enzyme activity in ES cells was determined by specific cleavage of the fluorescent substrate Ac-DEVD-AMC (27). Cells (1×10^7) were lysed in cell lysis buffer (50 mM HEPES, pH 7.4, 0.1% Chaps, 5 mM DTT, 0.1 mM EDTA) for 5 min at 4°C and centrifuged for 10 min at $10{,}000 \times g$. The protein content was subsequently determined with the Bradford assay. Total protein (10 μg) was added to 80 μl assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% Chaps, 10 mM DTT, 1 mM EDTA, 10% glycerol) and to 10 μl of the caspase-3 substrate Ac-DEVD-AMC, providing a final

Table 1. Primers Used for qRT-PCR.

Gene	Primer Sequence
Insulin	F: 5′-CCCACCCAGGCTTTTGTCAAACAGC-3′ R: 5′-TCCAGCTGGTAGAGGGAGCAGATG-3′
Glucagon	F: 5′-CAGGGCACATTCACCAGCGACTAC-3′ R: 5′-TCAGAGAAGGAGCCATCAGCGTG-3′
Somatostatin	F: 5′-ATGCTGTCCTGCCGTCTCCA-3′ R: 5′-TGCAGCTCCAGCCTCATCTCG-3′
IAPP	F: 5′-TGCAGCTCCAGCCTCATCTCG-3′ R: 5′-CTCTCTGTGGCACTGAACCA-3′
Glut2	F: 5′-GAAGACAAGATCACCGGAACCTTGG-3′ R: 5′-GGTCATCCAGTGGAACACCCAAAA-3′
Glucokinase	F: 5′-GAGGTCGGCATGATTGTGGGCA-3′ R: 5′-GCGCCCCACTCTGTGTTGACACAC-3′
Kir6.2	F: 5′-TGCTGTCCCGAAAGGGCATTATC-3′ R: 5′-TGCAGTTGCCTTTCTTGGACACG-3′
Sur1	F: 5′-ACCAAGGTGTCCTCAACAACGGCT-3′ R: 5′-TGGAGCCAGGTGCTATGGTGAATG-3′
Nestin	F: 5′-GAGAGTCGCTTAGAGGTGCA-3′ R: 5′-CCACTTCCAGACTAAGGGAC-3′
NCAM	F: 5′-CGACGAGGCCGAATACGTCTG-3′ R: 5′-GCTCCTCTAGTTCCATGGCCGTC-3′
Pdx1	F: 5′-ACCGCGTCCAGCTCCCTTTC-3′ R: 5′-CAACATCACTGCCAGCTCCACC-3′
Nkx6.1	F: 5′-AGAACCGCAGGACCAAGTGGAGAA-3′ R: 5′-TCGTCATCCTCCTCATTCTCCGAAG-3′
Oct4	F: 5′-AGGCCCGGAAGAGAAAGCGAACTA-3′ R: 5′-TGGGGGCAGAGGAAAGGATACAGC-3′
Cytokeratin 19	F: 5′-GGTGCCACCATTGACAACTC-3′ R: 5′-CTGCATCTCCAGGTCAGTCC-3′
Carbonic anhydrase 2	F: 5′-CCACCACTGGGGATACAGCAAGC-3′ R: 5′-GTCCTCCTTTCAGCACTGCATTGTC-3′
Amylase 2	F: 5′-CTGTGAACACAGATGGCGTCAAATC-3′ R: 5′-GCAGGAAGACCAGTCTGTAAAGTGGC-3′
Albumin	F: 5′-CCTCCTCTTCGTCTCCGGCTCTG-3′ R: 5′-GGGATTTGTCACAGTTGGCGGC-3′
β-Actin	F: 5′-AGAGGGAAATCGTGCGTGAC-3′ R: 5′-CAATAGTGATGACCTGGCCGT-3′

F: forward (sense) primer; R: reverse (antisense) primer.

substrate concentration of 0.03 mM. The increase of free fluorescence was quantified fluorimetrically at 360 nm (excitation) and 460 nm (emission) for 3 h at 37°C. Caspase-3 activity was measured throughout the increase of fluorescence in 60 min. The units were calculated against a standard dilution curve of free AMC. The caspase-3 enzyme activity is expressed as one unit = cleavage of 1 nmol AMC $\times$ h^{-1}.

Implantation of ES Cells

CD-1 albino mice (Charles River, Margate, UK) were housed in an air-conditioned room at 21 ± 1°C and 50%

humidity with a 12:12 h light/dark cycle. Drinking water and a standard breeding diet (RM3, Special Diet Services, Witham, UK) were freely available. Seventeen male mice at 10–15 weeks of age were used in the experiments. Diabetes was induced by IP administration of streptozotocin (120 mg/kg) to 4-h fasted mice. Blood glucose was monitored (Glucotrend, Roche Diagnostics, Lewes, UK) using nonfasted tail-tip blood samples, and implants were undertaken after 7–12 days when blood glucose was typically 270–360 mg/dl. Food was withheld 2–4 h before surgery under isoflurane anesthesia. Implants of approximately 2×10^7 cells, corresponding to 66 ng insulin/2×10^7 cells for implants from the reference protocol and 96 ng insulin/2×10^7 cells for implants from the new optimized four-stage differentiation protocol, were introduced beneath the left kidney capsule. Blood glucose and body weight were monitored until the study was terminated. All animal procedures were conducted in accordance with the British Animals Scientific Procedures Act.

Alkaline Phosphatase Staining

For alkaline phosphatase staining, undifferentiated stem cells and cells differentiated in a spinner culture were trypsinized and seeded on a feeder layer of inactivated mouse embryonic fibroblasts and cultured for 4–5 days in stem cell medium. Subsequently the cells were stained for alkaline phosphatase with the alkaline phosphatase detection kit from Chemicon according to the manufacturer's instructions (Chemicon, Temecula, CA, USA). For light microscopy, stained colonies were observed on a Nikon TMS microscope using phase-contrast filters and documented with a Nikon Coolpix 4500 digital camera.

Insulin Content

For measurement of cellular insulin content, cells were sonicated in Krebs-Ringer buffer. Insulin was determined by ultrasensitive ELISA according to the manufacturer's instructions.

Ultrastructural Characterization

For electron microscopy, cell pellets were fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3, postfixed in 1% OsO_4, and finally embedded in Epon. Thin sections were contrast stained with saturated solutions of lead citrate and uranyl acetate and viewed in an electron microscope (12).

Statistical Analyses

Data are expressed as mean values ± SEM. Unless stated otherwise statistical analyses were performed using one-way ANOVA followed by Dunnett's test for multiple comparisons or *t*-test for correlations using the Prism analysis program (Graphpad, San Diego, CA, USA).

RESULTS

Comparison of Four Different Protocols for Differentiation of Embryonic Stem Cells Into Insulin-Producing Cells

Islet Hormones. Mouse embryonic stem (ES) cells did not express pancreatic islet hormones when maintained in an undifferentiated, pluripotent state cultured in the presence of LIF (Table 2). When the cells were differentiated according to the protocol by Lumelsky and coworkers (16) quantitative analysis of gene expression confirmed expression of the four endocrine hormones insulin, glucagon, somatostatin, and IAPP (Table 2). When the cells were differentiated according to the Lumelsky protocol in the additional presence of 5% FCS during the last 7 days of the 26-day protocol, the expression levels of the three major hormones insulin, glucagon, and somatostatin were reduced by around 50% (Table 2). Only the expression of IAPP was increased (Table 2). Thus, the addition of FCS, though it preserved the ultrastructure (data not shown) of the differentiated cells, neither reduced the polyhormonal character nor affected the expression level of the hormones positively (Table 2). This, however, was achieved through the new optimized four-stage differentiation protocol. The cells expressed insulin, while glucagon expression was not detectable and somatostatin expression was lower than the Lumelsky reference protocol (Table 2). Even the expression of IAPP was significantly reduced (Table 2).

Structural Markers. Mouse ES cells did not express the Glut2 glucose transporter gene and ES cells showed virtually no expression of the neuronal cell markers nestin and NCAM. The genes for glucokinase and the genes for Kir6.2 and Sur1, which are characteristic for insulin-secreting β-cells as well as neuronal cells, were expressed in ES cells, but to a lesser extent than in cells differentiated according to the Lumelsky reference protocol (Table 2).

Addition of 5% FCS during the last 7 days of differentiation culture of the Lumelsky protocol significantly increased expression of the Glut2, Sur1, and nestin genes in particular (Table 2). In the new optimized four-stage differentiation protocol the expression level of the three structural genes typical for β-cells, namely glucokinase, Kir6.2, and Sur1, were maintained at a high level while the expression of the neuronal markers nestin and NCAM decreased very significantly (Table 2). The expression level of the Glut2 glucose transporter was significantly increased. In addition, expression levels of

Table 2. Comparison of Four Protocols for Differentiation of Mouse Embryonic Stem (ES) Cells Into Insulin-Producing Cells

	ES Cells (%)	Lumelsky Protocol (%)	Lumelsky Protocol + FCS (%)	Optimized Four-Stage Protocol (%)	Spinner Culture (%)
Islet hormones					
Insulin	n.d. (4)*	100 ± 8 (16)	57 ± 3 (8)	131 ± 14 (30)	11 ± 3 (3)
Glucagon	n.d. (4)*	100 ± 23 (8)	41 ± 16 (3)	n.d. (4)*	n.d. (4)*
Somatostatin	n.d. (4)†	100 ± 20 (8)	42 ± 16 (4)†	11 ± 3 (21)†	2 ± 1 (3)†
IAPP	n.d. (4)†	100 ± 15 (10)	147 ± 31 (6)	66 ± 14 (16)	3 ± 0 (4)*
Structural markers					
Glut2	n.d. (4)	100 ± 21 (11)	239 ± 52 (5)	270 ± 53 (18)*	45 ± 15 (3)
Glucokinase	44 ± 7 (4)	100 ± 10 (10)	86 ± 7 (5)	105 ± 18 (21)	38 ± 3 (5)
Kir6.2	32 ± 3 (4)	100 ± 6 (8)	149 ± 28 (5)	103 ± 18 (18)	39 ± 19 (4)
Sur1	28 ± 4 (5)	100 ± 11 (11)	226 ± 32 (4)	175 ± 30 (21)	51 ± 3 (4)
Nestin	2 ± 0 (5)†	100 ± 16 (9)	357 ± 39 (3)†	47 ± 8 (21)†	30 ± 8 (4)*
NCAM	1 ± 0 (4)†	100 ± 15 (10)	54 ± 10 (5)*	17 ± 2 (17)†	51 ± 9 (3)*
CK19	146 ± 72 (4)	100 ± 22 (5)	275 ± 80 (3)	895 ± 81 (10)†	103 ± 18 (5)
CA2	30 ± 9 (4)	100 ± 29 (4)	20 ± 8 (3)	561 ± 103 (11)*	236 ± 77 (4)
Transcription factors					
Pdx1	554 ± 93 (4)	100 ± 21 (7)	140 ± 79 (3)	1246 ± 274 (19)*	335 ± 60 (3)
Nkx6.1	1 ± 0 (4)*	100 ± 13 (9)	253 ± 36 (4)†	71 ± 17 (19)	8 ± 2 (3)
Oct4	264 ± 34 (4)†	100 ± 10 (7)	265 ± 80 (4)†	138 ± 17 (21)	432 ± 25 (3)†

Data shown are gene expression values determined by qPCR on day 26 of differentiation according to the Lumelsky reference protocol, the Lumelsky protocol plus 5% fetal calf serum (FCS), the new optimized four-stage protocol, a spinner culture protocol, and, for comparison, the undifferentiated ES cells. Depicted are changes in relative gene expression in percent normalized to the reference protocol by Lumelsky. Values shown are means ± SEM of the relative gene expression with the numbers of experiments given in parentheses. n.d. = not detectable.
*$p < 0.05$, compared with differentiated cells according to the Lumelsky reference protocol (second column).
†$p < 0.01$, compared with differentiated cells according to the Lumelsky reference protocol (second column).

carbonic anhydrase 2 (CA2) and cytokeratin 19 (CK19), both markers for pancreatic duct cells, were increased significantly by ninefold and sixfold, respectively (Table 2). Gene expression analysis of amylase revealed only traces of mRNA expression in cells from the reference protocol, while in cells from the new optimized four-stage protocol and in stem cells amylase expression remained undetectable (data not shown). Albumin expression, a marker for the differentiation towards hepatic progeny, was also negligible in all protocols with levels typically at or below the detection limit of the qRT-PCR (data not shown).

Transcription Factors. In contrast to ES cells differentiated according to the Lumelsky protocol, undifferentiated ES cells did not express Nkx6.1, which is a transcription factor characteristic for developing CNS and insulin-producing cells (Table 2). However, ES cells showed expression of the embryonic transcription factor Oct4 and the β-cell transcription factor Pdx1 (Table 2). Addition of 5% FCS to the culture medium of the Lumelsky protocol increased expression in particular of Oct4 and Nkx6.1 (Table 2). Differentiation according to the new optimized four-stage protocol did not significantly affect Nkx6.1 and Oct4 expression when compared to the Lumelsky reference protocol but increased expression of Pdx1 very significantly (Table 2).

Spinner Culture. ES cells, which were differentiated in a spinner culture flask, exhibited negligible signs of expression of endocrine marker genes (Table 2). The gene with the highest expression was Oct4, which is known to be increased at the beginning of a differentiation process for embryonic stem cells (Table 2). Nkx6.1 expression remained marginal and Pdx1 expression was also not increased when compared to undifferentiated pluripotent ES cells (Table 2). A significant expression of nestin and NCAM during spinner culture when compared to undifferentiated pluripotent ES cells indicated a differentiation towards neuronal progeny (Table 2), but not towards endocrine lineage as illustrated by the low expression levels of insulin, somatostatin and IAPP (Table 2). Indeed, the expression of glucagon could not be detected in cells from the spinner culture. There was low-level expression of other structural genes, such as Glut2, glucokinase, Kir6.2, and Sur1, after spinner culture, generally similar to undifferentiated pluripotent ES cells (Table 2). Only the expression of CA2 was elevated around twofold when compared with ES cells, but this increase was not significant (Table 2).

Effect of Serum Supplementation on Insulin Gene Expression in Mouse ES Cells After Differentiation According to the Lumelsky Reference Protocol

Insulin gene expression of ES cells after differentiation with increasing amounts of fetal calf serum (1–10%) during the last 7 days of the differentiation protocol caused a significant decrease in the expression of insulin (Fig. 2A). Insulin gene expression decreased to 40% compared to the Lumelsky reference protocol, when the culture medium had been supplemented with 5% FCS (Fig. 2A). Higher FCS concentrations up to 10% did not further decrease insulin gene expression (Fig. 2A).

Effect of Insulin Removal From the Culture Medium on Insulin Gene Expression in ES Cells After Differentiation According to the Lumelsky Reference Protocol

When the medium was deprived of insulin, expression of the insulin gene decreased to 74% compared to the reference protocol, which was supplemented with 25 μg/ml insulin (Fig. 2B).

Changes of the Ultrastructure of ES Cells Undergoing Differentiation

ES cells that were differentiated according to the Lumelsky reference protocol possessed typical features of neuroendocrine cells. These cells contain a large number of small dark peptidergic vesicles presumably containing neurotransmitters and neuropeptide hormones. These cells were often detected adjacent to nerve fibers (Fig. 3A). In contrast, ES cells that were differentiated according to the new optimized four-stage protocol exhibited clear signs of endocrine differentiation (Fig. 3B). These cells showed both a well-developed rough endoplasmic reticulum and Golgi apparatus, well suited for insulin synthesis, together with a large number of insulin-containing secretory granules, some located in the close vicinity of the plasma membrane appropriate for exocytosis (Fig. 3B).

Comparison of the Caspase-3 Activity of ES Eells, Embryoid Bodies, and Cells at Days 12, 19, and 26 of Differentiation According to the Lumelsky Reference Protocol or to the New Optimized Four-Stage Protocol

Undifferentiated ES cells exhibited low caspase-3 activity (Fig. 4). Upon differentiation and formation of embryoid bodies (EBs) the enzyme activity increased around fourfold. When the cells were selected according to the Lumelsky reference protocol towards nestin-positive cells, the caspase-3 activity increased around ninefold at day 12 compared with undifferentiated ES cells. After a similar period cells differentiated by the new optimized four-stage protocol showed a significantly lower caspase-3 activity (Fig. 4). During the nestin expansion step of the Lumelsky protocol caspase-3 activity decreased but was still about twofold higher than in cells differentiated with the new optimized four-stage protocol (Fig. 4). After 26 days of differentiation cells from both protocols possessed comparable caspase-3 activity levels (Fig. 4).

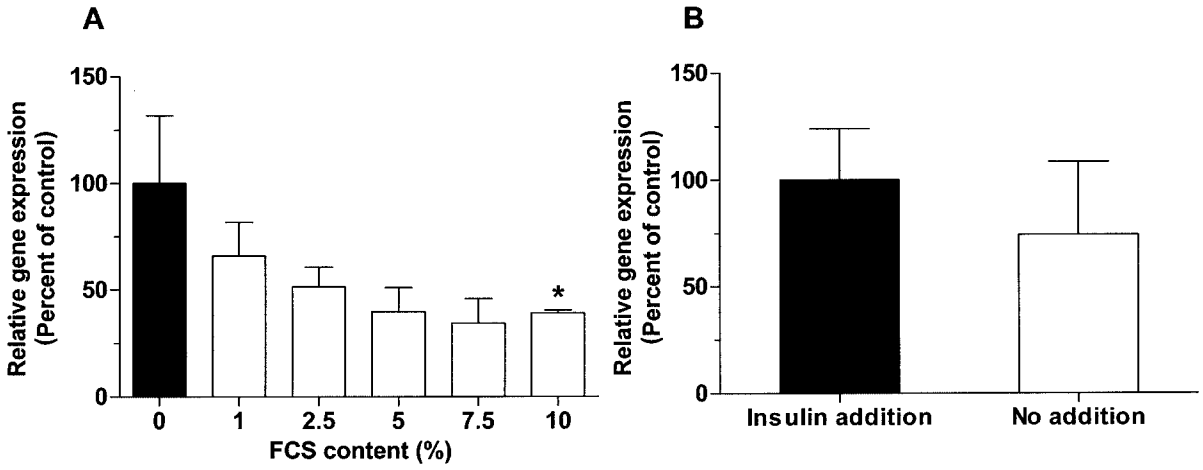

Figure 2. (A) Effect of serum supplementation on insulin gene expression in mouse embryonic stem (ES) cells after differentiation according to the Lumelsky reference protocol. (B) Effect of insulin removal from the differentiation medium on the insulin gene expression in ES cells after differentiation with the Lumelsky reference protocol. Data shown are insulin gene expression values determined by qPCR on day 26 of differentiation. Depicted are the changes in relative expression in percent normalized to the Lumelsky reference protocol. Values shown are means ± SEM of the relative gene expression of five to seven experiments. $*p < 0.05$ compared with cells cultured with 0% FCS.

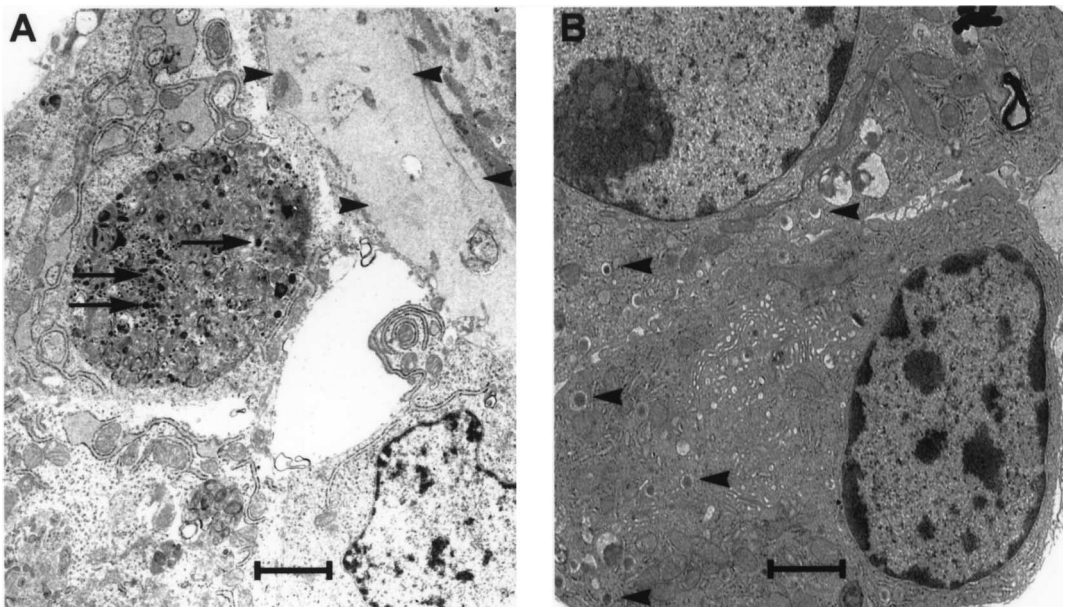

Figure 3. Changes of the ultrastructure of mouse embryonic stem (ES) cells undergoing differentiation. (A) Ultrastructure of a cell with neuroendocrine characteristics differentiated according to the Lumelsky reference protocol. The electron micrograph shows a neuroendocrine cell with peptidergic vesicles (arrow) in close proximity to a nerve fiber (arrowhead). Scale bar: 1 μm. (B) Ultrastructure of an insulin-producing cell differentiated according to the new optimized four-stage protocol. The electron micrograph shows an endocrine cell with insulin secretory granules (arrowhead) with a halo and dense core region. Scale bar: 1 μm.

Ultrastructure and Alkaline Phosphatase Staining of Undifferentiated Mouse ES Cells and ES Cells After 26 Days in a Spinner Culture

Analysis of ES cells by electron microscopy showed typical structural features of undifferentiated ES cells. The large nucleus typically appeared irregularly shaped with a high number of dense nucleoli (Fig. 5A). The cells exhibited a low ratio of cytoplasm to nucleus and a modest development of cellular organelles such as rough endoplasmatic reticulum (ER) and Golgi apparatus, although the cytoplasm was rich in free ribosomes and mitochondria. The cells frequently displayed microvilli leaving an obvious extracellular space between adjacent cells. Cell contacts between neighboring cells such as gap junctions were not observed (Fig. 5A).

ES cells, which were differentiated in a spinner culture, generally displayed the same phenotype as undifferentiated ES cells (Fig. 5B). The ratio of cytoplasm to nucleus was low. In addition the cytoplasm was rich in free ribosomes and mitochondria, as shown by undifferentiated ES cells (Fig. 5B). The cells formed cell aggregates with 50–70 cells in one section without gap junctions or other cell contacts (Fig. 5B). Morphological signs of neuroendocrine and especially endocrine differentiation could not be observed in cells from the spinner culture.

Undifferentiated ES cells grown on a layer of mitotically inactivated fibroblasts appeared upon light microscopical examination as typically round-shaped colonies. The colonies consisted of several hundred cells with uniform red staining of alkaline phosphatase that was slightly stronger at the periphery of a single colony than the core (Fig. 5C).

When cells from the spinner culture were replated on an inactivated feeder layer, they underwent continuous growth in distinct colonies. The colonies increased in size without any changes in the phenotype or signs of differentiation (Fig. 5D). The outgrowth retained the appearance of round-shaped colonies with smooth edges. Upon alkaline phosphatase staining these colonies showed the same red staining pattern as undifferentiated ES cells (Fig. 5D).

Implantation of Mouse Embryonic Stem (ES) Cells Differentiated According to the Lumelsky Reference Protocol and the New Optimized Four-Stage Protocol Under the Kidney Capsule of Streptozotocin (STZ) Diabetic Mice

The streptozotocin (STZ)-induced diabetes was associated with a progressive increase of hyperglycemia (Fig. 6). Without treatment (control), STZ-diabetic mice developed severe hyperglycemia (>400 mg/dl) and health status deteriorated, requiring this group to be terminated at day 14. Pseudoislets comprising about 2 ×

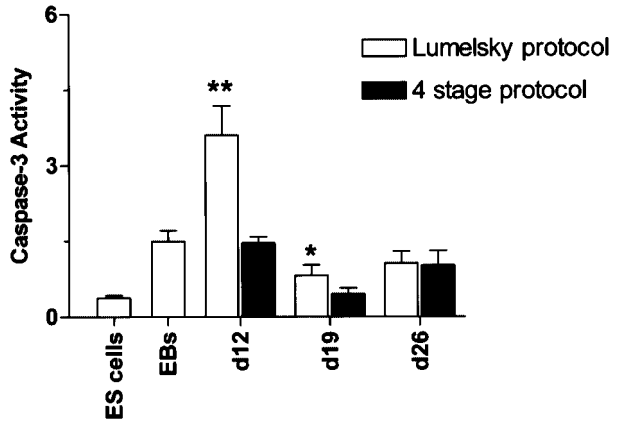

Figure 4. Comparison of the caspase-3 activity of mouse embryonic stem (ES) cells, EBs and cells at days 12, 19, and 26 of differentiation with the Lumelsky protocol or the new optimized four-stage protocol. Values shown are means ± SEM of the caspase-3 activity (μmol DEVD × h^{-1}) of 6–14 experiments. *$p < 0.05$, **$p < 0.01$ comparison between the Lumelsky and the new optimized four-stage protocol.

10^7 cells differentiated by the Lumelsky reference protocol were implanted under the kidney capsule. These pseudoislets were transferred into insulin-free buffered saline prior to implantation to avoid any acute glucose-lowering effect of the high insulin concentration in the differentiation medium. The pseudoislet implant did not prevent the rise in hyperglycemia, but limited the blood glucose concentration to about 400 mg/dl for 3–16 days after implantation. In contrast to the control and Lumelsky reference groups, mice implanted with 2×10^7 cells differentiated by the optimized four-stage differentiation protocol prevented the rise in hyperglycemia, and reduced blood glucose concentrations to <200 mg/dl by day 16. Cells were implanted using insulin-free buffered saline to avoid any artifactual effect of insulin in the medium.

Insulin Content

Content of insulin protein, determined by ELISA, was in the same range when cells had been differentiated in the presence of exogenous insulin according to the Lumelsky reference protocol (3.08 ± 0.09 pg insulin/μg DNA) and according to the new optimized four-stage protocol (4.68 ± 1.10 pg insulin/μg DNA). Removal of exogenous insulin during the last 7 days of differentiation culture reduced the cellular insulin content. However, this reduction was significantly more with the Lumelsky reference protocol (0.33 ± 0.24 pg insulin/μg DNA) than with the new optimized four-stage protocol (2.67 ± 0.74 pg insulin/μg DNA). In undifferentiated ES cells and cells subjected to 26 days of spinner culture insulin content values were below the detection limit.

DISCUSSION

In this study we developed a new optimized four-stage protocol for differentiation of mouse ES cells towards insulin-producing cells and compared this new differentiation protocol with a reference protocol originally developed by Lumelsky and collaborators (16). In addition we studied the effect of FCS supplementation to the culture medium and analyzed the potential suitability of a spinner culture technique for differentiation of ES cells towards insulin-producing cells. We reproduced the protocol by Lumelsky and coworkers and, in contrast to earlier work (25), we obtained evidence for insulin gene expression along with the expression of other pancreatic islet hormones. However, analysis of neuronal markers exhibited a high expression level of the nestin and NCAM genes, while expression of Pdx1, which is required for transactivation of the insulin gene, was lower than the Pdx1 expression detected in undifferentiated ES cells (17). Furthermore, withdrawal of exogenous insulin from the differentiation medium markedly lowered the insulin content of these cells, even though insulin gene expression was not decreased significantly. This could in part explain the confusion in previous studies regarding the source of the insulin accumulated by the cells, and explain why the original Lumelsky reference protocol has created a false impression of effectiveness (13,20,23,25).

The lack of Pdx1 expression and the inability to store significant quantities of insulin despite clearly detectable insulin expression indicate a nonregulated activation of the insulin gene, such as reported for developing tissue of the central nervous system (7–9,22). Indeed, ultrastructural analyses revealed that the differentiated cells exhibited typical signs of neuronal development. Not surprisingly, therefore, these cells failed to significantly reduce blood glucose concentrations in the STZ-diabetic mouse model. Thus, the gene expression pattern found in cells of the Lumelsky protocol (16) together with the ultrastructural findings and the in vivo results provide clear proof for differentiation towards a neuronal cell type with a moderate insulin expression but without typical characteristics of insulin-producing β-cells.

Because the original protocol proposed the idea of nestin positivity as a crucial feature of a putative β-cell precursor cell, the nestin selection step was introduced into the Lumelsky protocol. We, however, observed that during the nestin selection within the Lumelsky differentiation protocol the activity of the apoptosis-inducing protein caspase-3 was prominent. Thus, the nestin selection step during differentiation of ES cells is apparently a means to enrich neuronal cells or those cells that are undergoing differentiation towards neuronal development, while differentiation towards cell types of the en-

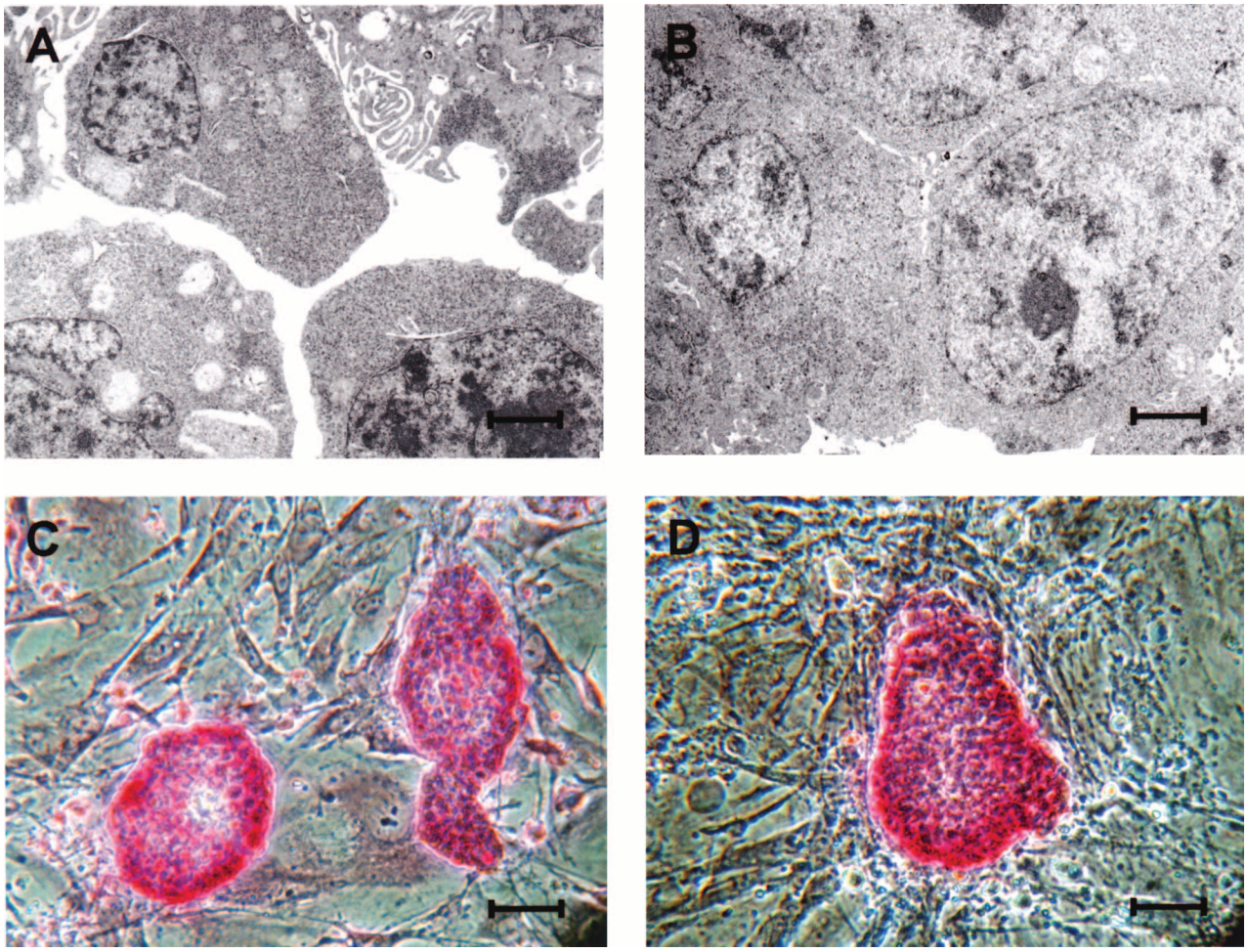

Figure 5. Ultrastructure (A, B) and alkaline phosphatase (C, D) staining of undifferentiated mouse embryonic stem (ES) cells (A, C) and ES cells after 26 days in a spinner culture (B, D). (A) Ultrastructure of undifferentiated ES cells showing free ribosomes and mitochondria but no other higher cell organelles. Scale bar: 1 μm. (B) Ultrastructure of undifferentiated cells after 26 days in a spinner culture with comparable morphology. Scale bar: 1 μm. (C) Alkaline phosphatase staining (red) in colonies of undifferentiated ES cells plated on a layer of inactivated feeder cells. Scale bar: 100 μm. (D) Alkaline phosphatase staining (red) in colonies of replated undifferentiated cells on a layer of inactivated feeder cells after prior spinner culture for 26 days. Scale bar: 100 μm.

docrine lineage may be suppressed. FCS supplementation to the original Lumelsky protocol even enhanced the neuronal character of the cells obtained with this differentiation protocol.

To overcome the drawbacks of the reference protocol we developed an improved differentiation protocol in order to obtain preferentially insulin-producing cells. We removed the nestin selection step and composed differentiation media lacking cell culture supplements that promote differentiation towards neuronal cells. In addition, the differentiation medium was supplemented with nutrients to avoid deprivation during the final cultivation step. We observed that cells that were differentiated according to this new optimized four-stage differentiation protocol expressed nearly exclusively insulin. Glucagon expression disappeared and somatostatin was negligible compared with the reference protocol. The reduced expression of nestin and NCAM reflects a shift away from neuronal differentiation towards a monohormonal population of insulin-producing cells. Interestingly, the removal of the nestin selection step also significantly reduced the activity of caspase-3, providing evidence for improved survival conditions of differentiated cells. At the same time the β-cell-like character of these insulin-producing cells was reinforced by the high expression of the Glut2 glucose transporter gene and other β-cell characteristic structural genes such as glucokinase, Kir6.2, and Sur1. Markers for differentiation towards ductal cells, namely CK19 and CA2, were significantly increased and the lack of expression of amylase and albumin excludes significant differentiation towards exocrine pancreas and hepatic progeny.

It is widely accepted that β-cells arise from ductal cells during organogenesis of the pancreas. Not surpris-

ingly, therefore, the strong expression of CK19 in our cells appears in parallel to the reinforced levels of insulin expression and other structural markers for endocrine cells. This reflects an in vitro differentiation mechanism analogous to that described for the differentiation of ductal tissue into endocrine insulin-producing cells during fetal development of the pancreas (19). Pdx1 is required for a regulated insulin expression in insulin-producing cells. In addition, we achieved a very high level of Pdx1 gene expression in cells differentiated according to this new optimized differentiation protocol.

We further investigated whether the changes in the expression pattern of these genes contributed to a phenotype with closer resemblance of a pancreatic β-cell. Indeed, the removal of the nestin selection step yielded cells with a clear endocrine phenotype as documented by electron micrographs of insulin-producing cells that contained insulin secretory granules. Addition of insulin to the differentiation medium of our new optimized differentiation protocol enabled the cells to produce and store more insulin independent of uptake of exogenous insulin from the differentiation medium. These findings were further supported by the outcome of the in vivo experiments, where implantation of cells from the new differentiation protocol prevented the rise of blood glucose in STZ-diabetic mice, and caused a significant reduction of the glucose values well below 200 mg/dl towards a range typical for nondiabetic mice.

It has recently been reported that a differentiation procedure based on the protocol by Lumelsky et al. (16) followed by a differentiation stage where adherent cells were transferred to a histotypic spinner culture produced a 14-fold increase in insulin content (3). The spinner culture keeps the cells in suspension during the whole differentiation process whereas the new optimized four-stage protocol presented in this study and other differentiation protocols developed earlier depend on adherent cell culture. In contrast to the previous report, we found that the spinner culture was not suitable for differentiation of ES cells towards insulin-producing cells, but rather maintained the embryonic character of the cells as documented ultrastructurally and by alkaline phosphatase staining of the cells (5). Moreover, we confirmed that the gene expression pattern in these cells demonstrated no significant changes when compared with undifferentiated ES cells.

This lack of differentiation during the spinner culture may be due to the inability of the cells to develop cell–cell contacts and gap junctions, which we confirmed by ultrastructural studies, because these are apparently required for differentiation (28). This prevention of differentiation caused by the prevention of adherence may represent an attractive method for standard tissue culture of embryonic stem cells, especially of those cell lines that normally require a layer of inactivated primary embryonic fibroblasts such as human embryonic stem cells. In conclusion, therefore, the present study has provided a new protocol for steering ES cells away from a neuronal progeny in order to facilitate differentiation to insulin-producing cells with β-cell-like features. The new protocol produced cells that can prevent the rising hyperglycemia after implantation in an animal model of

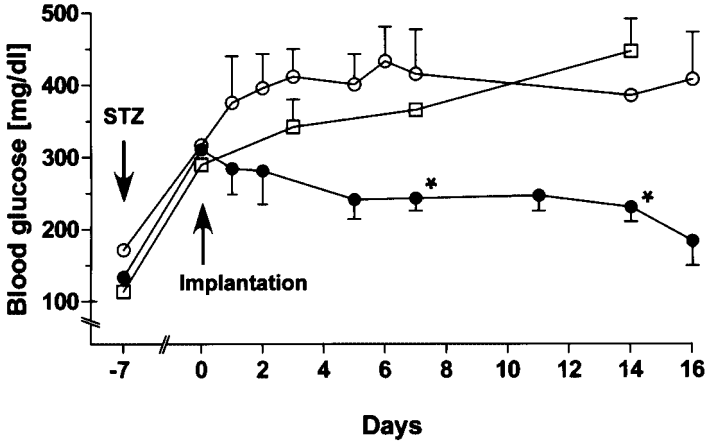

Figure 6. Implantation of mouse embryonic stem (ES) cells differentiated according to the Lumelsky reference protocol and the new optimized four-stage protocol under the kidney capsule of streptozotocin (STZ) diabetic mice. Shown are blood glucose concentrations of diabetic mice injected with streptozotocin (STZ) (120 mg/kg) 7 days prior to implantation of cells differentiated according to the Lumelsky reference protocol (open circles) or to the new optimized four-stage protocol (filled circles) and for comparison of control STZ diabetic mice that received no cells (open boxes). Values shown are means ± SEM from four to seven animals. $*p < 0.05$ compared with cells from the Lumelsky reference protocol.

insulinopenic diabetes. We also present evidence that preventing cell adherence can prolong the pluripotent embryonic state.

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