Changes in gene expression and morphology of mouse embryonic stem cells upon differentiation into insulin-producing cells *in vitro* and *in vivo*

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Abstract

Background

A comparative morphological analysis of *in vitro* differentiated and *in vivo* implanted mouse ES cells towards insulin-producing cells has not been studied before. The impact of the *in vivo* environment on behavior of ES cells after implantation is of major importance for a potential cell replacement therapy of type 1 diabetes mellitus.

Methods

ES cells differentiated *in vitro* into insulin-producing cells according to the Lumelsky protocol or a new 4 stage differentiation protocol were analysed before and after implantation for gene expression by *in situ* RT-PCR, protein expression by immunohistochemistry, and by ultrastructural analysis.

Results

In comparison with undifferentiated and nestin positive ES cells developed according to the reference protocol, the number of ES cells differentiated with the new 4 stage protocol increased substantially under *in vivo* conditions. The cells, grown in a tissue-like structure, exhibited, in comparison to the *in vitro* situation, increased gene and protein expression of Pdx1, insulin, IAPP, the GLUT2 glucose transporter and glucokinase, which are functional markers for glucose-induced insulin secretion of pancreatic beta cells. Renal sub-capsular implantation of ES cells with a higher degree of differentiation achieved by *in vitro* differentiation with a new 4 stage protocol enabled further significant maturation for beta the cell specific markers insulin and the co-stored IAPP as well as the glucose recognition structures GLUT2 glucose transporter and glucokinase. In contrast, a further *in vivo* differentiation was not achieved with cells differentiated *in vitro* by the reference protocol.

Conclusions

Thus a sufficient degree of *in vitro* differentiation, with the development of organelles for the protein synthesis apparatus, is an essential prerequisite for further substantial maturation in a beta cell specific way under tissue-like conditions *in vivo*, supported by cell-cell contacts and vascularisation.

Introduction

Implantation of insulin-producing cells into a diabetic host is a promising therapeutic option for the treatment of diabetes [1]. The limited availability of donor islets for transplantation has drawn attention to embryonic stem cells, which are available in unlimited numbers and have the potential to differentiate into most somatic cell types, including insulin-producing cells [2]. While different groups have recently published advances in this field [3,4], the morphology of mouse ES cells differentiated *in vitro* towards insulin-producing cells has not been analysed in detail before and after implantation. In particular the impact of the in vivo environment on differentiation and maturation of differentiated ES cells after implantation has not been analysed yet. Therefore, it was the aim of this study to examine the maturation process and the benefit of the in vivo environment including 3-dimensional growth in a tissue-like manner. We therefore performed a morphological study on ES cells which were differentiated *in vitro* according to a reference protocol previously published by Lumelsky and collaborators [5] and to new efficient 4 stage differentiation protocol [6]. Differentiated cells from both protocols were implanted into CD-1 albino mice and grafts were explanted 14 days after implantation.

The present *in vitro* transition from ES cells into insulin-producing cells during differentiation was documented using a detailed analysis of protein expression of beta cell markers and embryonic transcription factors. Moreover, the cells from both protocols were subjected to an ultrastructural analysis of cell organelles to assess the differentiation status of the cells. Grafts of implanted ES cells were analyzed in parallel for gene and protein expression using the same beta cell markers and embryonic transcription factors as under *in vitro* conditions.

Pdx1 is an important marker for beta cell development and is expressed from the earliest stage of pancreas development when the epithelial duct forms. Later, Pdx1 is selectively expressed in mature beta cells. Since Pdx1 is the major transactivator of the insulin gene its presence is decisive for the transcriptional regulation of the insulin gene [7].

Downstream of the transcription and translation of the insulin gene there is processing of the proinsulin protein into mature insulin by cleavage of the C-peptide and storage in secretory granules. This maturation process is associated with the storage of insulin in the core and C-peptide into in the outer halo region of secretory granules. Thus, C-peptide is a suitable marker to show the processing of proinsulin into mature insulin and to verify de novo synthesis of insulin (to exclude the possibility of insulin uptake from an exogenous source) [8]. IAPP was used as a second islet hormone marker because this peptide is specifically

expressed in pancreatic beta cells without the possibility of uptake from the culture medium [9].

Two additional structures, which are essential for signal recognition in the process of glucose-induced insulin secretion of beta cells, are the GLUT2 glucose transporter and the glucose phosphorylating enzyme glucokinase. Glucokinase is the glucose sensor of the beta cell, coupling glucose recognition to the exocytosis of insulin [10] [11,12]. The GLUT2 glucose transporter allows unlimited intracellular access of glucose to glucokinase, which is transactivated by Pdx1 [13]. Therefore the expression of Pdx1 together with insulin and C-peptide, the co-stored IAPP as well as the GLUT2 glucose transporter and glucokinase, were analysed to monitor the benefit of the *in vivo* environment on differentiated stem cells. In order to analyze the loss of stemness during the course of differentiation and subsequent implantation, the gene and protein expression of the embryonic marker genes Oct4 and Nanog were monitored. The expression of the intermediate neurofilament protein nestin was measured to assess whether neuronal progeny can be reduced with the new differentiation protocol in comparison to the reference protocol. This study shows that ES cells differentiated *in vitro* with the new 4 stage protocol comprised a higher degree of differentiation and continued to maturate further *in vivo* when compared to those of the reference protocol.

Materials and methods

Materials

DMEM and DMEM/F-12 tissue culture media, glutamine, non-essential amino acids and bFGF 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 synthesised 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 dNTP's were from Genecraft (Münster, Germany). Unless otherwise mentioned chemicals of analytical grade were obtained from Sigma or Merck (Darmstadt, Germany).

Cell culture conditions

The mouse embryonic stem (ES) cell line ES-D3 [14] 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 non-essential amino acids, 0.1 mM β-mercaptoethanol, 50 µg/ml gentamicin and 1000 U/ml LIF in a humidified atmosphere at 37°C with 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 according to a reference protocol [5] or to a new optimized 4 stage differentiation protocol [2,6]. For differentiation with the new optimized 4 stage differentiation protocol the cells were trypsinized and counted with a hemocytometer. 10^6 cells were transferred to 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 embryoid bodies 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 non-essential 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 µM non-essential amino acids and 10 mM nicotinamide.

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 12:12h light/dark cycle. Drinking water and a standard breeding diet (RM3, Special Diet Services, Witham, UK) were freely available. Male mice were used at 10-15 weeks of age. Diabetes was induced by i.p. administration of streptozotocin (120 mg/kg) to 4 h fasted mice. Blood glucose was monitored (Glucotrend^{R}, Roche Diagnostics, Lewes, UK) using non-fasted tail-tip blood samples, and implants were undertaken after 7-12 days when blood glucose was typically 270 – 360 mg/dl. Food was withheld 2-4h before surgery under isoflurane anaesthesia. Implants of approximately 2 x 10^7 cells were introduced beneath the left kidney capsule. Mice were killed by cervical dislocation after 14 days and the implants were removed for histological and immunocytochemical analyses. All animal procedures were conducted in accordance with the British Animals Scientific Procedures Act.

Tissue processing

For light microscopy, ES cells immobilized on slides, ES cell pellets and transplanted ES cells beneath the kidney capsule were fixed in 4 % para-formaldehyde in 0.15 M phosphate buffered saline (PBS), pH 7.3, and embedded in paraffin. Additionally, small ES cell pellets were fixed in 2 % para-formaldehyde and 2.5 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3, postfixed in 1 % OsO_4 and finally embedded in Epon for electron microscopic analysis [15].

Immunocytochemical staining of slides

For immunocytochemical staining of ES cells, 1 x 10^6 differentiated cells were seeded overnight on glass slides and subsequently fixed with ice cold methanol. After a washing step the cells were permeabilized and blocked with PBS plus 0.2 % Triton X-100 and 5 % donkey serum. The slides were incubated with primary antibodies [Table 2] diluted in PBS with 0.1 % Triton X-100 and 0.1 % BSA at room temperature for 30 min or overnight at 4°C. Following this step the cells were washed with PBS and incubated with secondary antibodies for 30 min (1:500). Secondary antibodies from donkey were conjugated with Cy2, Texas Red or Cy5 obtained from Dianova (Hamburg, Germany). For nuclear counterstaining 300 nM DAPI was used for 5 min at room temperature. Slides were thereafter mounted with Mowiol (Merck, Darmstadt, Germany) plus 0.6 % Dabco (St. Louis, MO, USA).

Immunohistochemical staining of sections

Serial paraffin sections of the ES cell pellets from the differentiation protocols and after transplantation under the kidney capsule were stained either by the avidin-biotin-complex (ABC) method [16] or by an immunofluorescence staining [16]. The slides were incubated overnight with the specific antibodies (Table 2], followed by a 30 min incubation with either biotinylated or fluorescence labelled goat anti-rabbit IgG, goat anti-guinea pig or rabbit anti-mouse IgG (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA). In the ABC method the slides were incubated with a mixture of streptavidin (1:100) and biotin-peroxidase (1:1000) (Jackson ImmunoResearch, Suffolk, UK) and developed with 0.7 mM diaminobenzidine in 0.002 % hydrogen peroxidase containing phosphate buffered saline (PBS), pH 7.3. The same primary antibodies were used as for the immunofluorescence staining of the slides (Table 2]. All antibodies were certified for immunohistochemistry and showed specific immunostaining in tissue sections from normal mouse pancreas or ES cells in early differentiation stages. In order to verify the changes in the densities of insulin protein expression in ES cells differentiated either according to the reference or the new 4 stage protocol in tissue like structures after implantation immunoreactivities were densitometrically quantified. A computer-assisted method (cell P software, Olympus) using bright field illumination with the BX61 upright microscope (Olympus Optical) was employed. The mean grey values per pixel of the protein expression ranged between 15 and 85 for all intact ES cells. For each animal (n = 4 in each group) protein expression data were expressed from four optical fields per section. Additionally the areas of the ES cell implants from the new 4 stage protocol which were positively immunostained for insulin were morphometrically determined.

In situ RT-PCR

Transplanted ES cells were fixed on 3-Chamber SuperFrost Plus™ slides on a heating block at 100 °C for 2 min. Subsequently the slides were treated with proteinase K (20 µg/ml) for 20 min at 37 °C. Proteinase K was inactivated thereafter at 95 °C for 2 min followed by an overnight incubation with a RNase-free DNase solution (1 U/ml) at 37 °C. The RT *in situ* PCR procedure was a modification of published protocols [17,18]. After rinsing with DEPC water and air drying reverse transcription was performed in a buffer containing a mixture of all nucleotides (1 mM dATP, dGTP, dCTP and dTTP, Genecraft, Germany), oligo-dT primer (Invitrogen, UK), M-MLV reverse transcriptase (0.5 U/µl) (Invitrogen, UK), RNasin (Promega, Madison, WI, USA) and ddH_2O. Slides were incubated at 37 °C for 1 h in a moist

chamber and thereafter reverse transcriptase was inactivated at 92 °C for 2 min. PCR amplification was performed in a buffer containing a nucleotide mix (10 µM digoxigenin (DIG) 11-dUTP, 190 µM dTTP, 200 µM each of dATP, dCTP, dGTP), the specific forward and reverse primer (1.25 µM) and *Taq* polymerase (0.1 U/µl) (Biotherm, Genecraft, Germany), self-seal reagent (MJ Research, Waltham, MA, USA) and ddH_2O. 15 µl of the PCR reaction mix was applied to each chamber of the slide and sealed by cover slips. Thereafter the slides were placed in a thermal cycler suitable for *in situ* amplifications (MJ Research, Waltham, MA, USA). PCR amplification was performed according to the following protocol: initial denaturation at 95 °C for 3 min, followed by 35 – 40 cycles with a denaturation at 95 °C for 45 seconds, annealing at 57 °C for 45 seconds and extension at 72 °C for 45 seconds. A final extension was performed for 10 min at 72 °C. After removal of the cover slips the slides were incubated with a blocking reagent for 1 h. Thereafter the incorporated DIG-labeled nucleotides were detected by an anti-digoxigenin antibody labeled with alkaline phosphatase (1:500) or rhodamine (1:300) at room temperature for 1 h. Alkaline phosphatase activity was detected by the NBT/BCIP (nitro blue tetrazolium chloride/ 5-bromo-4-chloro-3-indolyl phosphate toluidine salt) color reaction (three hours incubation at room temperature). The primer sequences for mouse preproinsulin, IAPP, glucagon, somatostatin, Pdx1, Oct4, nestin, GLUT2, GK, ß-actin were listed in Table 1.

Analysis of ES cells

Immunostained ES cells mounted on cover slips were examined with an IX81 inverted microscope (Olympus Optical, Tokyo, Japan). Sections of ES cell pellets or ES cell grafts stained for gene or protein expression were viewed using bright field and fluorescence illumination with the BX61 upright microscope (Olympus Optical). Both microscopes were equipped with specific excitation filter systems for rhodamine, Cy2, fluorescein isothiocyanate (FITC), Texas Red, Cy5 and DAPI dyes (AHF Analysentechnik, Tübingen, Germany). Ultrathin sections for ultrastructural analysis of ES cells were viewed by an electron microscope 10 (Carl Zeiss AG, Oberkochen, Germany). To identify growing behaviour under culture conditions the cells were observed with a Nikon TMS microscope using phase-contrast filters and documented with a Nikon Coolpix 4500 digital camera.

Results

Phase contrast analysis of cell morphology in vitro

Undifferentiated mouse ES cells, when observed under light microscopy, grew in typically spherical and smooth colonies of variable size (Figure 1A, D]. Individual cells were difficult to distinguish within the embryonic stem cell colonies, except for the nuclei comprising normally one or more nucleoli. Stem cell colonies were thickened in the center but thinned out towards the periphery of the colony. Some ES cells at the periphery were stretched and formed bulky pseudopodia [Figure 1A, D].

Upon differentiation and nestin selection according to the reference protocol, the typical stem cell colony morphology disappeared after the intermediate embryonic body formation step and the majority of cells acquired a distinct neuronal-like cell appearance [Figure 1B, E]. These cells formed complex branched networks with axonal characteristics. Those colony-forming networks were often attached to a fibroblast-like cell layer [Figure 1B, E].

When embryonic stem cells were cultured according to the new 4 stage differentiation protocol, which is devoid of the nestin selection step, the cells grew out to form a monolayer without any signs of neuronal-like differentiation and with cuboid, as well as fibroblast-like cell types. At the end of the differentiation process three-dimensional buds branched out of these monolayer cells and were evenly distributed (Figure 1C, F]. These cell clusters were composed of cells with distinguishable cell borders and without any of the neuronal-like branches observed in the reference protocol. The colonies seen with undifferentiated stem cells were virtually absent from this time point.

In situ PCR of ES cells in vitro

Using the in situ RT-PCR technique, gene expression of peptides and transcription factors was analysed in ES cells differentiated according either to the reference protocol or to the new 4 stage protocol. In ES cells differentiated according to the reference protocol, mRNA transcripts of preproinsulin were absent in intact cells, but were detected in the cytoplasm of intact ES cells developed according to the new 4 stage protocol [Table 3]. The majority of these ES cells revealed gene expression of the peptide IAPP which is naturally co-stored in mature beta cell insulin secretory granules. The gene expression of other islet hormones was detected to a minor degree in non-insulin positive cells from both differentiation protocols [Table 3]. Pdx1 mRNA expression was not detected in the cytoplasm of ES cells differentiated according to the reference protocol, while significant expression of Pdx1 was found in the cytoplasm of intact ES cells differentiated according to the new 4 stage protocol [Table 3]. The mRNA transcripts of the glucose recognition structures, namely the GLUT2 glucose transporter and glucokinase were found in single ES cells of the new 4 stage protocol, but were not detected in cells from the reference protocol [Table 3]. Conversely, mRNA expression of the intermediate neurofilamentous protein nestin was visible in nearly all ES cells from the reference protocol [Table 3], but markedly decreased in ES cells from the new 4 stage protocol [Table 3]. A strong reduction of nestin mRNA expression was observed in ES cells using the new 4 stage protocol together with a reduction of the transcription factors Nanog and Oct4. This demonstrates the higher degree of differentiation achieved using the 4 stage protocol compared with the reference protocol.

Immunocytochemical staining of ES cells in vitro

ES cells cultured according to the reference protocol showed very dense staining for insulin in apoptotic cells lacking an intact nucleus [Figure 2A]. Mostly, these ES cells expressed no marker for insulin biosynthesis. In contrast, ES cells differentiated according to the new 4 stage differentiation protocol showed positive immunostaining for C-peptide and insulin with a dot-like appearance in the cytoplasm. This was evident in single cells [Figure 2B] and in pseudoislet aggregates after 26 days of culture (data not shown). Staining for the neuroectodermal marker nestin was present in the majority of the cells differentiated towards the neuronal fate in the Lumelsky protocol. These cells showed a strong filamentous immunostaining in the cytoplasm [Figure 2C], whereas the ES cells developed according to the new 4 stage protocol revealed a much reduced number and lower intensity of nestin positive cells [Figure 2D]. Remarkably, a distinct population of densely immunostained nestin

positive cells from the reference protocol showed inclusion bodies immunoreactive for insulin in the cytoplasm (see also the ultrastructural analysis results). This staining was different from the dot-like appearance found in cells from the new 4 stage differentiation protocol. The immunostaining for the embryonic transcription factors Oct4 and Nanog revealed a small clustered subpopulation of undifferentiated stem cells which showed a nuclear co-staining for both transcription factors in cells from both differentiation protocols [Figure 2 E and F].

Ultrastructure of undifferentiated and differentiated ES cells in vitro

Ultrastructural analysis of ES cells revealed the typical morphology of undifferentiated cells. The nuclei were large and unevenly shaped, with a high number of dense nucleoli [Figure 3A]. The ratio of cytoplasm to nucleus was low, and higher cell organelles, such as rough endoplasmic reticulum (ER) and Golgi apparatus, were poorly developed. The cytoplasm was rich in free ribosomes and mitochondria [Figure 3A]. Despite the densely packed morphology of the stem cell colonies, specialized cell contacts such as gap junctions were not observed. Stem cells located in the periphery of colonies often showed microvilli, thereby defining extracellular space between proximate colonies

Differentiated ES cells, which were developed according to the reference protocol, showed neuroectodermal characteristics as depicted in Figure 3B. Some cells displayed a fiber phenotype with a high amount of neurofilaments and a small number of other cell organelles such as mitochondria, characteristic of axonal regions (Figure 3B]. Other well differentiated cells contained high numbers of peptidergic vesicles concentrated in dense body formations, sometimes surrounded by a cellular membrane. Other regions of these cells showed well developed rough endoplasmic reticulum and mitochondria

In contrast to the neuronal differentiation of cells from the reference protocol, ES cells from the new 4 stage protocol exhibited clear characteristics of endocrine differentiation with respect to subcellular organelles for synthesis, processing, storage and release of preproinsulin without concomitant signs of neuronal differentiation [Figure 3C]. The cells showed a well developed rough endoplasmic reticulum and Golgi apparatus, consistent with substantial protein synthesis and the formation of secretory granules. This was complemented with a high number of mitochondria providing the basis for sufficient energy supply for these processes. Many of these granules demonstrated typical features of beta cell insulin granules with a distinctive dark core region and a pale surrounding halo region [Figure 3C].

In situ PCR of ES cells after implantation in a diabetic mouse model

Using the *in situ* RT-PCR technique, gene expression of peptides and transcription factors were analyzed in differentiated ES cells grown in a graft with a tissue-like character after implantation. Oct4 mRNA transcripts as a sign of remaining pluripotency were exclusively found in specific regions in the ES cells from grafts of the reference protocol [Figure 4A]. In contrast, ES cells of the grafts differentiated according to the new 4 stage protocol mostly lost the Oct4 gene expression in the *in vivo* situation 14 days after implantation [Figure 4B]. In opposite, Pdx1 mRNA expression was rarely detected in the cytoplasm of the ES cell graft differentiated according to the reference protocol [Figure 4C], while abundant Pdx1 gene expression was found in the cytoplasm of intact ES cell grafts differentiated according to the new 4 stage protocol [Figure 4D]. Conversely, the mRNA of intermediate neurofilamentous protein nestin was homogeneously expressed in nearly all ES cells from the reference protocol [Figure 4E]. However, gene expression of nestin was markedly decreased within ES cell grafts from the new 4 stage protocol in the cytoplasm cells [Figure 4F].

In ES cell grafts differentiated according to the reference protocol mRNA transcripts of preproinsulin were rarely seen in the cytoplasm of intact cells. A significant level of preproinsulin gene expression was observed mostly ~~only~~ in apoptotic cells [Figure 5A]. After differentiation according to the new 4 stage protocol, preproinsulin mRNA was expressed in cytoplasmic regions in the ES cell graft. Most importantly, these mRNA transcribing ES cells showed no signs of apoptosis [Figure 5B]. Additionally, these ES cells also revealed a high mRNA expression of the peptide IAPP which is naturally co-stored in mature beta cell secretory granules [Figure 5D]. ES cell grafts from cells differentiated according to the reference protocol expressed mRNA for IAPP at a lower degree which may represent also early stages of glucagon-producing cells [Figure 5C]. Gene expression for GLUT2 glucose transporter was markedly increased in the ES cell grafts of the new 4 stage protocol [Figure 5F] compared with the reference [Figure 5E]. The glucokinase mRNA expression which was not detectable in the cytoplasm of ES cells differentiated according to the reference protocol [Figure 5G] showed a dense signal in the cytoplasm of ES cells of the new protocol grafted after for 14 days *in vivo* [Figure 5H].

Protein expression of embryonic transcription factors in ES cells after implantation in a diabetic mouse model

Changes in the degree of differentiation were studied after implantation of differentiated ES cells into diabetic mice. The embryonic transcription factors Oct4 and Nanog were

immunohistochemically analysed in cells from both protocols. Only ES cells from the reference protocol showed distinct nuclear staining [Table 4]. Positive cells were mostly clustered and located in highly apoptotic areas. Interestingly, after implantation immunostaining of Oct4 in cells from the new 4 stage protocol was only found in the cytoplasm with very faint staining and in only a minor portion of the cells [Table 4]. The immunostaining pattern of Nanog was similar to Oct4 [Table 4].

Protein expression of beta cell markers in ES cells after implantation in a diabetic mouse model

In order to verify the higher *in vivo* maturation of the ES cells of the new 4 stage protocol the protein expression of beta cell markers was assessed. Some ES cells from the reference protocol showed a dense immunoreactivity for insulin in the cytoplasm but these cells also showed morphological signs of apoptosis [Figure 6A and Table 4].

ES cells differentiated according to the new 4 stage protocol exhibited a distinct C-peptide and insulin positive staining and were organized in a structured tissue [Figure 6B]. Densitometrical measurements of insulin immunoreactivity from intact cells of the reference compared to new 4 stage protocol revealed an 3.6 fold increase in densities (17 $\pm$ 4 to 61 $\pm$ 7, mean grey value per pixel). Additionally the areas which were positively immunostained for insulin and C-peptide were the less than 3 % of the whole implant in cells from the Lumelsky protocol (n = 4 in each group), whereas in cells from the new 4 stage protocol the area ranged between 22 to 35 % of the whole implant with a mean value of 26 $\pm$ 3 %. Other islet hormones could not be traced immunohistochemically [Table 4]. Glucokinase protein expression was not detected in the cytoplasm of ES cells in the reference protocol [Figure 6C] but showed a distinct cytoplasmic staining in intact cells differentiated according to the new 4 stage protocol [Figure 6D]. Only apoptotic ES cells from the reference protocol stained positively for the GLUT2 glucose transporter [Figure 6E]. The immunostaining of GLUT2 was found in the cytoplasm and rarely seen in the plasma membrane [Figure 6F]. The results from the *in vivo* experiments are summarized in Table 4.

Discussion

In this study we compared the morphological changes of mouse ES cells after differentiation into insulin-producing cells *in vitro* and after subsequent implantation *in vivo*. The cultured ES cells were analysed immunohistochemically and ultrastructurally. Grafts of implanted ES cells were analyzed for expression of transcription factors and beta cell marker genes, both on the gene and protein level by *in situ* RT-PCR and immunohistochemistry. The aim of this study was to assess the effect of implantation into a diabetic animal and to document the effect of this environment on the differentiation efficiency using two different protocols. We developed a new optimized 4 stage protocol for differentiation of mouse ES cells towards insulin-producing cells [6] and compared this protocol with a reference protocol originally designed for neuronal differentiation but later adopted for differentiation of ES cells towards endocrine cell types [5].

In vitro differentiation

When ES cells were differentiated under *in vitro* conditions with either the reference or the new protocol the cells lost the typical stem cell phenotype characterized by a smooth surface, non-subdivision of cells, a syncytium-like formation and colony growth [19]. Throughout the final days of differentiation the cells from the reference protocol developed axonal branches to a high degree, confirming earlier reports [20]. In contrast, cells from the new 4 stage protocol lacked this axonal appearance, providing no indication for differentiation towards the neuronal lineage. The cells from the new 4 stage protocol formed a two-layered tissue with a basal monolayer from which buds of cell clusters grew out. Recent work in the field of adult stem cell differentiation has provided evidence that those cell clusters might serve as a pool for endocrine progenitor cells under *in vitro* conditions [21].

The suppression of neuronal differentiation was verified by the loss of nestin immunoreactivity in cells from the new protocol, whereas nestin positive cells formed the major population of cells differentiated by the reference protocol. Remarkably, the reduction of nestin expression was paralleled by an increase of cells positive for insulin and C-peptide after the new differentiation protocol. The beneficial reduction of nestin was consistent with recent findings from another new differentiation protocol for mouse ES cells [22], but in contrast to previous reports from the same group [23] and other investigators [24]. These cells were viable and showed no signs of apoptotic or necrotic cell death. The immunohistochemical results with respect to nestin expression were confirmed in the present study by ultrastructural analyses showing distinct organelles for protein biosynthesis in cells

from the new protocol and moderate insulin staining. In agreement with the results of the present study, other groups have recently shown that such a reduction of neuronal cell fate is a prerequisite for efficient differentiation into endocrine or other somatic cell types [3,25,26].

In vivo maturation

Nonetheless, it has not been shown to which extent the *in vivo* situation can foster the maturation process of endocrine cells derived from ES cells. In particular the present study has examined the loss of embryonic transcription factor expression and functional characteristics after implantation of insulin-producing cells. Other groups have shown that blood glucose concentrations of diabetic animals could be lowered by implantation of differentiated insulin-producing ES cells. In those studies the morphology of the ES cell graft was only analysed by conventional HE staining or immunostaining for insulin [22,24,25,27-29]. Such studies did not establish whether an insulin-producing capability was further improved and sustained after implantation. The present study documents a further decrease of embryonic transcription factors and in parallel an increase of crucial beta cell markers at the gene and protein expression level.

After implantation of ES cells from both protocols the beneficial effect of the *in vivo* environment was visible by a distinct reduction of Oct4 and Nanog expressing cells. This was associated with clear signs of apoptosis, which would permit the removal of undifferentiated cells in the grafts of the implanted ES cells. Implanted ES cells from the reference protocol were somewhat protected against cell death that usually occurred at this stage but showed no development of typical beta cell markers. In contrast, ES cells differentiated *in vitro* according to the new 4 stage protocol showed high expression of the crucial Pdx1 transcription factor. Pdx1 is the major regulator of beta cell development during pancreas organogenesis and continues to act in adult cells as a regulatory element for insulin gene expression [7,30,31]. Thus, an important milestone in the differentiation of ES cells towards the lineage of endocrine insulin-producing cells is the presence of the gene and protein expression of Pdx1, which has only been documented previously in cultures of *in vitro* differentiated human ES cells [3,4] or in one recent study *in vivo* [32]. In cells from the reference protocol, Pdx1 expression remained virtually undetectable even after implantation, indicating the failure of that differentiation protocol to proceed to true insulin-producing cells [8,20,33]. However, cells from the new protocol showed Pdx1 expression together with mRNA transcription of the preproinsulin gene and mRNA encoding proinsulin. In addition expression of IAPP was detected normally co-stored together with insulin in mature secretory granules of beta cells.

This excludes the possibility that the insulin could derive only from uptake of insulin from the extracellular space [9] thereby providing strong evidence of 'proper' endocrine differentiation. In grafts of ES cells differentiated prior to implantation according to the reference protocol, the expression of the GLUT2 glucose transporter and the glucose phosphorylating enzyme glucokinase, which are both prerequisites for regulated release of insulin, was only detected in single cells and with very faint intensity. The higher differentiation level of implanted insulin-producing cells originating from the new 4 stage protocol comprised the gene and protein expression of both glucose recognition structures and thus leading eventually to a correction of blood glucose levels in STZ diabetic mice [6]. The formation of teratomas after implantation is a crucial safety concern when considering cell replacement therapies with differentiated ES cells. It is well known that undifferentiated ES cells reside in differentiation cultures and will form teratomas or teratocarcinomas after implantation even if efficient *in vitro* differentiation protocols have been used [32]. The removal of ES cells by suicide gene expression or cell selection using fluorescence activated cell sorting (FACS) has to be combined with differentiation techniques and is an important prerequisite for future studies. Taken together, the gene and protein expression of the hormones and the glucose recognition structures, plus Pdx1 clearly demonstrate the beneficial effect of the *in vivo* environment for a directed differentiation towards endocrine insulin-producing cells with characteristics comparable to those of pancreatic beta cells. However, a 'proper' and efficient protocol for differentiation of ES cells towards insulin-producing cells is a crucial prerequisite for further maturation after *in vivo* implantation. This benefit of a favourable *in vivo* environment is negligible, however, unless appropriate differentiation towards insulin-producing cells is established during the preceding *in vitro* phase of differentiation.

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Conflict of interest

None declared.

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Tables

Table 1 Primer sequences used for *in situ* PCR

Gene		Primer sequence
Insulin	Fw	5’-CCCACCCAGGCTTTTGTCAAACAGC-3’
	Rv	5’-TCCAGCTGGTAGAGGGAGCAGATG-3’
IAPP	Fw	5’-TGCAGCTCCAGCCTCATCTCG-3’
	Rv	5’-CTCTCTGTGGCACTGAACCA-3’
Glucagon	Fw	5’-CAGGGCACATTCACCAGCGACTAC-3’
	Rv	5’-TCAGAGAAGGAGCCATCAGCGTG-3’
Somatostatin	Fw	5’-ATGCTGTCCTGCCGTCTCCA-3’
	Rv	5’-TGCAGCTCCAGCCTCATCTCG -3’
Nestin	Fw	5’-GAGAGTCGCTTAGAGGTGCA-3’
	Rv	5’-CCACTTCCAGACTAAGGGAC-3’
GLUT2	Fw	5’-GAAGACAAGATCACCGGAACCTTGG-3’
	Rv	5’-GGTCATCCAGTGGAACACCCAAAA-3’
Pdx1	Fw	5’-ACCGCGTCCAGCTCCCTTTC-3’
	Rv	5’-CAACATCACTGCCAGCTCCACC-3’
Glucokinase	Fw	5’-GAGGTCGGCATGATTGTGGGCA-3’
	Rv	5’-GCGCCCCACTCTGTGTTGACACAC-3’
Oct4	Fw	5’-AGGCCCGGAAGAGAAAGCGAACTA-3’
	Rv	5’-TGGGGGCAGAGGAAAGGATACAGC-3’
Beta actin	Fw	5’-AGAGGGAAATCGTGCGTGAC-3’
	Rv	5’-CAATAGTGATGACCTGGCCGT-3’

Fw – forward (sense) primer; Rv – reverse (antisense) primer. If possible amplicons were designed intron-spanning and were in a size ranging from 100-300 bp. Beta actin was used as housekeeping gene.

Table 2 Antibodies for immunohistochemical staining

Antibody	Working Dilution	Source
Rat insulin, guinea pig	1:200	Abcam, Cambridge, UK
Rat insulin, guinea pig	1:600	Dako, Hamburg, Germany
Human IAPP, rabbit	1:500	Peninsula, San Diego, USA
Rat c-peptide, goat	1:1000	Linco Research, St. Charles, USA
Human glucagon, rabbit	1:2000	Dako, Hamburg, Germany
Human somatostatin, rabbit	1:1000	Abcam, Cambridge, UK
Mouse nestin, mouse	1:200	Chemicon, Temecula, USA
Mouse GLUT2, goat	1:500	Santa Cruz Biotechnology, Santa Cruz, USA
Mouse, Pdx1, mouse	1:200	R&D Systems, Minneapollis, USA
Rat Glucokinase, rabbit	1:50	Own production, affinity purified
Mouse Oct4, mouse	1:200	R&D Systems, Minneapollis, USA
Mouse Nanog, rabbit	1:200	Abcam, Cambridge, UK

Table 3 Changes in gene and protein expression of transcription factors, endocrine cell and beta cell specific markers in ES cells differentiated with the reference protocol or the new 4 stage differentiation protocol under *in vitro* conditions.

Parameter	**Reference protocol**		**4 stage protocol**	
	Gene expression	Protein expression	Gene expression	Protein expression
Insulin	0	0	(+)	(+)
Glucagon	+	+	0	0
Somatostatin	+	+	(+)	(+)
IAPP	0	0	(+)	(+)
Pdx1	+	+	++	(+)
GLUT2	0	0	(+)	(+)
Glucokinase	0	0	(+)	(+)
Nestin	+++	++	+	(+)
Nanog	+++	++	+	(+)
Oct4	+++	++	+	(+)

The score represents the following stages: 0 = no expression, (+) = in single cells, + = in up to 5-10 % of the intact cells, ++ = 11-25 % of the cells, +++= in more than 50 % of cells. * gene expression found by *in situ* PCR.

Table 4 Changes in gene and protein expression of transcription factors, endocrine cell and beta cell specific markers in ES cells differentiated with the reference protocol or the new 4 stage differentiation protocol under *in vivo* conditions.

Parameter	**Reference protocol**		**4 stage protocol**	
	Gene expression	Protein expression	Gene expression	Protein expression
Insulin	(+)	(+)	++	+
Glucagon	+	++	(+)	(+)
Somatostatin	+	+	0	0
IAPP	(+)	(+)	++	+
Pdx1	+	+	+++	+
GLUT2	+	+	++	++
Glucokinase	(+)	(+)	++	++
Nestin	+++	++	(+)	0
Nanog	+++	++	(+)	(+)
Oct4	+++	++	(+)	(+)

The score represents the following stages: 0 = no expression, (+) = in single cells, mostly with apoptotic or necrotic signs, + = in up to 5-10 % of the intact cells, ++ = in 11-25 % of the cells, +++ = in more than 50 % of cells.

Legends to figures

Figure 1. Phase contrast analysis of undifferentiated ES cells and differentiated ES cells after differentiation with the reference protocol or the new 4 stage differentiation protocol *in vitro*. Morphology of undifferentiated ES cells with typical colony appearance (*arrows*) and pseudopodia (*arrowhead*) (**A**, **D**), ES cells differentiated according to the reference protocol with axonal-like branches (*arrowheads*) on a monolayer of fibroblasts (*arrows*) (**B**, **E**) and to the new 4 stage differentiation protocol with formation of a monolayer (*arrows*) and buds of cell clusters (*arrowheads*) (**C**, **F**) in two different magnifications.

Figure 2. Immunocytochemical staining of ES cells after differentiation according to the reference protocol or to the new 4 stage differentiation protocol. ES cells differentiated according to the reference protocol (**A**, **C**, **E**) and to our new differentiation protocol the (**B**, **D**, **F**) were fixed on glass slides and co-stained for C-peptide (green) and insulin (red) (**A**, **B**), nestin (green) and insulin (red) (**C**, **D**) and Oct4 (green) and Nanog (red) (**E**, **F**). The merge is shown in yellow. Original magnification 400x. Scale bar = 10 µM.

Figure 3. Ultrastructure of ES cells. Ultrastructure of undifferentiated ES cells (**A**), ES cells differentiated according to the reference protocol (**B**) and to our new 4 stage differentiation protocol (**C**). Differentiated ES cells after the reference protocol showed features of the neuroendocrine development as nerve fibers (*arrows*). ES cells after differentiation with the new protocol showed the features of an insulin-producing cell as beta cell typical secretory granules (*arrowheads*), a well developed rough endoplasmic reticulum and Golgi apparatus.

Figure 4. Gene expression in grafts of ES cells after differentiation with the reference protocol or the new 4 stage differentiation protocol. mRNA-transcripts encoding either the transcription factor Oct4 (**A, B)**, the beta cell transcription factor Pdx1 (**C, D**) and the intermediate filament protein nestin (**E, D**) were analysed by *in situ* RT-PCR in grafts of ES cell differentiated with either the Lumelsky protocol (**A, C, E**) or with the new 4 stage differentiation protocol (**B, D, F**). Dense Oct4 mRNA expression was detected in defined areas of the graft in ES cells differentiated with the reference protocol and was strongly reduced in the graft of the new 4 stage protocol. Pdx1 was highly expressed in ES cells differentiated with the new 4 stage protocol compared with cells from the reference protocol. Grafts of ES cells, which were differentiated with the new 4 stage protocol showed nestin mRNA expression only at a low degree, while cells from the Lumelsky protocol showed abundant nestin transcripts.

Figure 5. Gene expression of implanted ES cells after differentiation with the reference protocol or the new 4 stage differentiation protocol. mRNA-transcripts encoding either the endocrine hormones insulin (**A, B**) and IAPP (**C, D**) and the glucose recognition structures GLUT2 glucose transporter (**E, F**) and glucokinase (**G, M)** were analysed by *in situ* RT-PCR in grafts of ES cell differentiated with either the Lumelsky protocol (**A, C, E, G**) or with the new 4 stage differentiation protocol (**B, D, F, H**). Insulin and IAPP mRNA expression was densely stained in ES cells after differentiation with the new 4 stage protocol. The gene expression of the GLUT2 glucose transporter markedly increased in the ES cells of the new 4 stage protocol in comparison to the ES cells of the reference protocol. The gene expression of glucokinase was mostly found only in the ES cells of the new 4 stage protocol.

Figure 6. Protein expression of beta cell markers in ES cell grafts after differentiation with the reference protocol or the new 4 stage differentiation protocol and implantation in a diabetic mouse model. Comparison of the *in vivo* protein expression in ES cells of C-peptide (**A**, **B**), glucokinase (**C**, **D**) and GLUT2 (**E**, **F**) after implantation under the kidney capsule of STZ diabetic mice. The cells were differentiated according to the reference protocol (**A**, **C**, **E**) or according to the new 4 stage protocol (**B**, **D**, **F**). The beta cell specific marker C-peptide as well as glucokinase and the GLUT2 glucose transporter showed a moderate to dense immunostaining (*arrowheads*) in intact ES cells differentiated with the new 4 stage protocol. Immunostaining of glucokinase was absent and immunostaining of C-peptide and GLUT2 was restricted to apoptotic cells (*arrows*) in the ES cells developed according to the reference protocol.