ISOLATION AND CHARACTERIZATION OF MESENCHYMAL STEM CELLS FROM CULTURED HUMAN PANCREATIC ISLETS OF LANGERHANS

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1. SUMMARY

Replacement of insulin producing β-cells that reside within pancreatic islets represents an almost ideal treatment for patients with diabetes mellitus type 1. But, transplantation of pancreatic islets or the entire pancreas is limited by the lack of donor organs. Stem cell derived insulin producing β-cells represent an attractive alternative. The major goal of our research project is the *ex vivo* generation of insulin producing cells using adult human stem cells that express nestin. Nestin is an intermediate filament protein that was originally described as a marker for embryonic and adult stem / progenitor cells of the central nervous system. Recently it has been also proposed to be a potential stem cell marker in rodent and human islets of Langerhans. Role and function of nestin positive pancreatic cells during development or adult life however are not well defined and matter of controversial debates. The first aim of our research project was to isolate a pure population of nestin expressing cells from human pancreatic islets of Langerhans for further characterization. For this purpose single cell derived colonies were isolated from cultured human islets. These colonies were immortalized and in a second step selected for cells with highest nestin promoter activity using promoter targeted selection of nestin expressing cells. The second aim was to characterize these cell lines especially their stem cell properties and differentiation potential. We characterized them finally as potential mesenchymal stem cells (MSC) of pancreatic origin based on their expression of different stem cell markers including nestin together with the transcription factor Islet-1 (Isl-1) and their ability to differentiate *in vitro* into mesoderm lineages (adipocytes and osteoblast-like cells). We could also demonstrate that they are multipotential and capable of differentiation into albumin producing liver-like cells *in vivo* and cells with a pancreatic endocrine phenotype *in vitro*. Based on these observations, we wanted to test the hypothesis that human nestin expressing

MSC from bone marrow and adipose tissue could equally harbour the potential to differentiate into insulin producing cells *ex vivo*. We found that bone marrow and adipose tissue derived MSC are also able to differentiate into cells expressing various pancreatic endocrine genes *in vitro* including several crucial transcription factors as well as the islet hormones insulin and glucagon. As a limitation of the presented work, we were not yet able to generate functional cells that secrete insulin in response to glucose. But, we can show for the first time that nestin positive MSC isolated from human adult pancreas, bone marrow and adipose tissue represent stem / progenitor cells with the potential to induce pancreatic developmental genes. These cells may have the capacity to become insulin secreting cells if further manipulated and exposed to appropriate microenvironment.

2. INTRODUCTION

2.1. Diabetes mellitus

Diabetes mellitus is a debilitating metabolic disease caused by absent (juvenile or type 1) or insufficient (type 2) insulin production from β-cells. With an actual prevalence of 5.9 % diabetes is affecting 15.7 millions people in the USA and approximately 400'000 in Switzerland (data for CH calculated). Diabetes is associated with serious long-term complications, such as cardiovascular disorders, kidney disease and blindness. The actual treatment strategies for type 1 diabetes (5-10 % of all diabetics) are based on insulin replacement by several injections daily combined with meticulous blood glucose monitoring and life style adaptations. But even under best circumstances type 1 diabetic patients are faced with a therapeutic dilemma: A good metabolic control that is prerequisite in order to avoid long-term complications like retinopathy or nephropathy is associated with a high frequency of severe hypoglycaemia [1]. Pancreas and islet transplantation are not real treatment alternatives. First of all because of the lack of donor organs, but also because of important perioperative complications associated with pancreas transplantation and the long-term risk of chronic immunosuppression [2,3]. Insulin secreting cells generated from embryonic or adult stem cells could represent an attractive alternative [4].

2.2. Stem cells as a potential source for β-cell replacement

Both embryonic stem cells (ESC) and adult stem / progenitor cells have been shown to harbour the ability to induce insulin expression *ex vivo* when exposed to the appropriate stimuli [4]. ESC have the potential for almost unlimited supply of insulin producing cells [5] but there are considerable ethical concerns associated with the use of human ESC. The generation of individual, patient specific stem cell-derived β-cells for transplantation

purposes requires therapeutic cloning of human ESC. This is not yet possible. Cloning of embryonic stem cells is already accomplished in mice and other mammals [6]. The only paper so far that claimed to have cloned human ESC reported fabricated data [7]. Another problem is related to the enormous proliferative capacity of ESC and the risk for development of teratocarcinoma. Therefore, many scientists continue to work with adult stem / progenitor cells. Large body of evidence indicates that such adult stem / progenitor cells are present in the pancreas. They were described in pancreatic islets [8,9,10], pancreatic ducts [11], among the population of pancreatic acinar cells [12-14] and in adult or fetal pancreas without further specification [15-17]. Adult stem / progenitor cells with the potential to differentiate into insulin producing cells have been also found outside the pancreas namely in the liver [18,19], central nervous system [20], spleen [21], skin [22] and bone marrow [23-26] indicating a greater developmental plasticity of adult stem / progenitor cells than was previously appreciated. Although the mechanisms are poorly understood and the differentiation efficacy is quite limited, it seems that adult stem / progenitor cells within and outside the pancreas can be converted into insulin producing cells. In this context, they should follow at least in part the complex program of normal pancreas development.

2.3. Transcriptional regulation of pancreas development

The adult pancreas is a mixed organ that contains three different cell types: the ductal tree, the exocrine acini that produce digestive enzyme, and the endocrine islets of Langerhans. They are embedded within the exocrine tissue and consist of four distinct cell types: α-, β-, δ- and PP-cells, which produce the key hormones glucagon, insulin, somatostatin and pancreatic polypeptide, respectively (Figure 1). During embryogenesis, the pancreas develops as an outgrowth at the foregut endoderm from epithelial cells that branch into the surrounding mesenchyme and fuse to form finally the mature organ.

Interactions between these epithelial and mesenchymal cells direct then pancreatic development towards endocrine or exocrine fate [27]. The first step of pancreas formation needs the suppression of sonic hedgehog (SHH) signalling by factors like activin and fibroblast growth factors in pre-patterned, restricted areas of the endoderm [28,29] thereby allowing the induction of the transcription factor Ipf-1 that is prerequisite for pancreas development. Loss of function mutation of Ipf-1 results in pancreas agenesis in mouse and man [30,31]. After this initial period, the coordinated and tightly controlled activation of various crucial transcription factors including Isl-1, Ngn3, Pax4, Pax6, Nkx2.2 and Nkx6.1 orchestrates the development of the pancreas and pancreatic endocrine cells. Isl-1 is together with Ipf-1 one of the earliest pancreatic markers detected in the pancreatic anlage at embryonic day e9 in the mouse [32]. During pancreas development Isl-1 is expressed in mesenchymal and epithelial cells and has been demonstrated to be crucial for the differentiation of endocrine cells. Explants of fetal pancreas from Isl-1 knockout mice are not able to generate endocrine cells as compared to wildtype animals [32]. At day e9-9.5 Ngn-3 is induced, another important step toward pancreatic endocrine cells. Ngn3 has recently been shown to be involved in the development of all four endocrine cell types [33], and Ngn3 positive cells have been widely regarded as the real progenitors of hormone producing islet cells [34]. In the adult pancreas no Ngn-3 expression is detected. Pax4 and Pax6 are expressed both in the developing gut and in the adult pancreas. Pax6 is required for the generation of glucagon secreting α-cells [35], whereas Pax4 plays a role in the differentiation of insulin producing β-cells and somatostatin producing δ-cells [36]. Nkx2.2 was shown to be involved in the early development of pancreatic β-cells [37] and Nkx6.1 is expressed primarily in β-cells of the adult islets [38]. Finally in adult pancreas, Ipf-1 is induced and regulates the expression of insulin in differentiated β-cells.

Most of these transcription factors are sequentially and transiently expressed during development (Figure 2.). Although pancreas development has been partly deciphered by identification and characterization of many transcription factors little is known about their function and molecular mechanism of action.

Differentiation of stem / progenitor cells into insulin producing cells *in vitro* should follow at least the major part of this transcriptional program. Adult stem cells cultured *in vitro* however may face certain limitations due to the lack of an appropriate *in vivo* environment and some crucial *in vivo* factors.

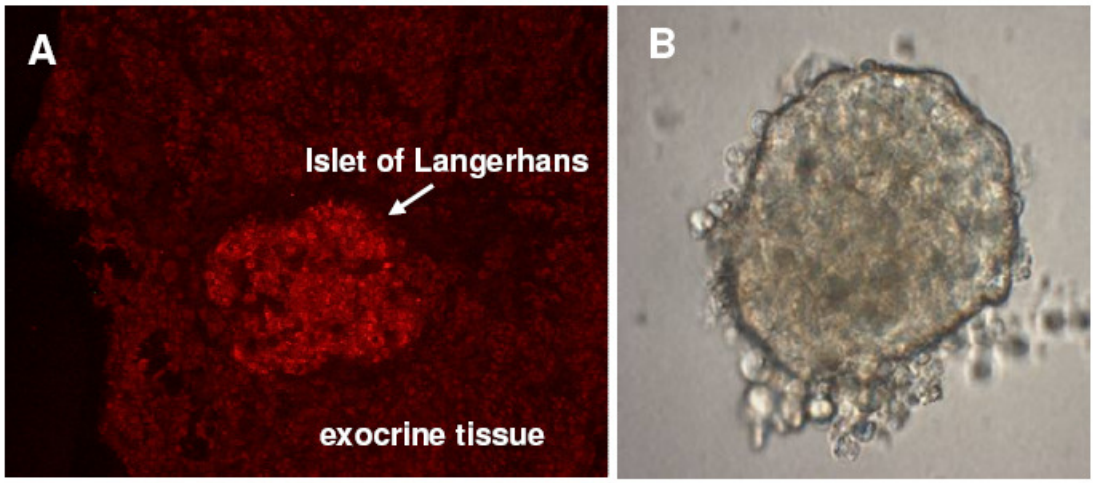

Figure 1.

Histology of the human pancreas. A. *An islet of Langerhans is surrounded by exocrine tissue (Immunostaining for C-peptide).* ***B.*** *Human islet cluster (phase contrast).*

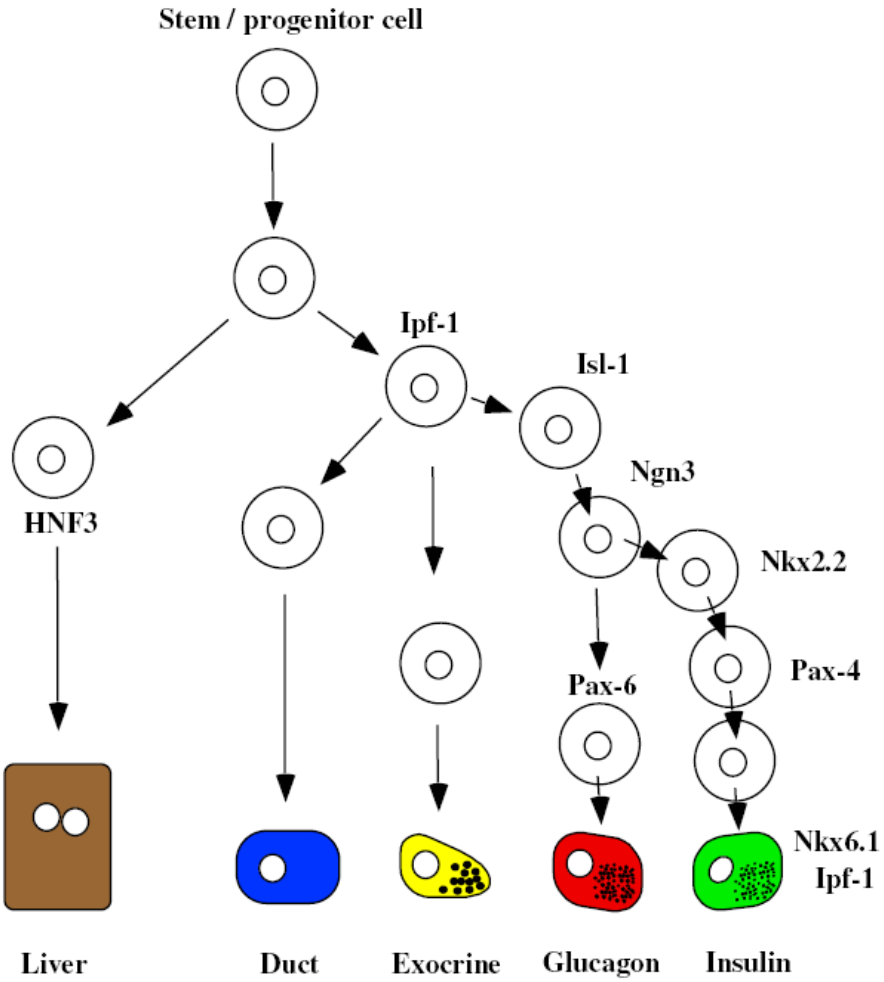

Figure 2.

Schematic representation of putative differentiation pathways leading from stem / progenitor cells to pancreatic β-cells. *The proposed model of the hierarchy of transcription factors is based on the temporal expression and phenotypic results of gene specific knockouts (after Schwitzgebel et al. 2001).*

3. MAIN FINDINGS AND GENERAL DISCUSSION

Cells in human pancreatic islets of Langerhans proliferate and start to express nestin when cultured in medium supplemented with serum and growth factors including FGF2 and EGF [8]. In order to show that nestin expressing cells have stem cell potential *in vitro* single cell cultures were isolated from cultured human islets. Single cell derived colonies were positive not only for nestin but also for Isl-1 whereas Ipf-1 as well as Insulin expression were negative. In order to ensure robust and sustained proliferation, cells were reversibly immortalized using CRE-Lox excisable lentiviral vectors over-expressing the hTelomerase and / or mBmi-1, a combination of genes which was described to be optimal for reversible immortalization of primary human cells [39] (Lentiviral transductions were performed by Patrick Salmon, University Hospital Geneva). These modified cell lines continued to express nestin and Isl-1. Whereas nestin is accepted as a marker of neural stem / progenitor cells, the pattern of expression and role of nestin during pancreas development remain controversial [40-47]. The transcription factor Isl-1 is known to play a crucial role in the development of endocrine pancreatic cells [32]. During embryogenesis, nestin and Isl-1 positive cells are found in the pancreatic mesenchyme surrounding the epithelial cells that equally express Isl-1 themselves [32,46]. In view of this co-expression of nestin and Isl-1 in pancreatic mesenchyme during development, we assumed to have isolated MSC from cultured pancreatic islets. This hypothesis was further supported by their ability to differentiate *in vitro* into mesoderm lineages like adipocytes (Figure 3) and osteoblast-like cells (Figure 4). In addition, these immortalized cells have the potential to acquire a hepatic phenotype *in* vivo when transplanted into the liver of immune deficient SCID mouse demonstrating for the first time *in vivo* that multipotential stem / progenitor cells may exist in human pancreatic islets (Figure 5). This part of the project was performed in

collaboration with Marc von Mach (University Hospital Mainz, Germany) and Jan Georg Hengstler (University of Leipzig, Germany). Similar results were obtained with a population of cultured nestin expressing human islet cells. They were also able to engraft into SCID mouse liver and produced human albumin 3 weeks after transplantation (Figure 6). The mechanisms underlying this phenomenon seem to be transdifferentiation although fusion with host hepatocytes cannot be completely ruled out. These results support the idea of common hepato-pancreatic stem / progenitor cells that may reside within the pancreas as well as the liver. During embryogenesis, both organs are believed to origin from similar endodermal progenitor cells [48] and there is evidence indicating that such progenitors with the potential to generate liver cells from pancreatic cells and vice versa are still present in adult life [49-51,18,19].

Surprisingly, MSC isolated from human bone marrow were also able to differentiate into hepatocytes without evidence for cell fusion if xenografted directly into the rat liver [52] demonstrating that MSC are able to cross the mesodermal lineage and give rise to endoderm *in vivo*.

Using a new differentiation protocol for the generation of insulin producing cells from our immortalized pancreatic MSC resulted in expression or up-regulation of various key transcription factors like Ipf-1, Isl-1, Pax-4, Pax-6, Nkx2.2, Nkx6.1 and Ngn-3 as well as the islet genes insulin, glucagon and somatostatin (Figure 7). Our differentiation conditions included serum free medium enriched with B-27 supplement, N-2 supplement, exendin-4, nicotinamide, hepatocyte growth factor, activin-A and pentagastrin, all factors known to have beneficial effects on differentiation of stem / progenitor cells into insulin producing cells [10,53-55]. Although C-peptide containing granules were found as an indicator for de-novo insulin synthesis in some differentiated cells (Figure 7), they were not yet able to secret C-peptide in response to glucose indicating a certain level of developmental immaturity. De-immortalization of the islet derived MSC could render them more

susceptible to adopt an endocrine phenotype and may lead to insulin producing and secreting cells. (**Eberhardt M***, Salmon P*, von Mach MA, Hengstler JG, Brulport M, Linscheid P, Seboek D, Oberholzer J, Barbero A, Martin I, Müller B, Trono D and Zulewski H. *"Multipotential nestin and Isl-1 positive mesenchymal stem cells isolated from human pancreatic islets"*. **Biochem Biophys Res Commun** 2006; 345: 1167 – 1178) and (von Mach MA, Hengstler JG, Marc Brulport M, **Eberhardt M**, Schormann W, Hermes M, Prawitt D, Zabel B, Grosche J, Reichenbach A, Müller B, Weilemann LS and Zulewski H. *"In vitro cultured islet-derived progenitor cells of human origin express human albumin in SCID mouse liver in vivo"*. **Stem Cells** 2004; 22: 1134 – 1141)

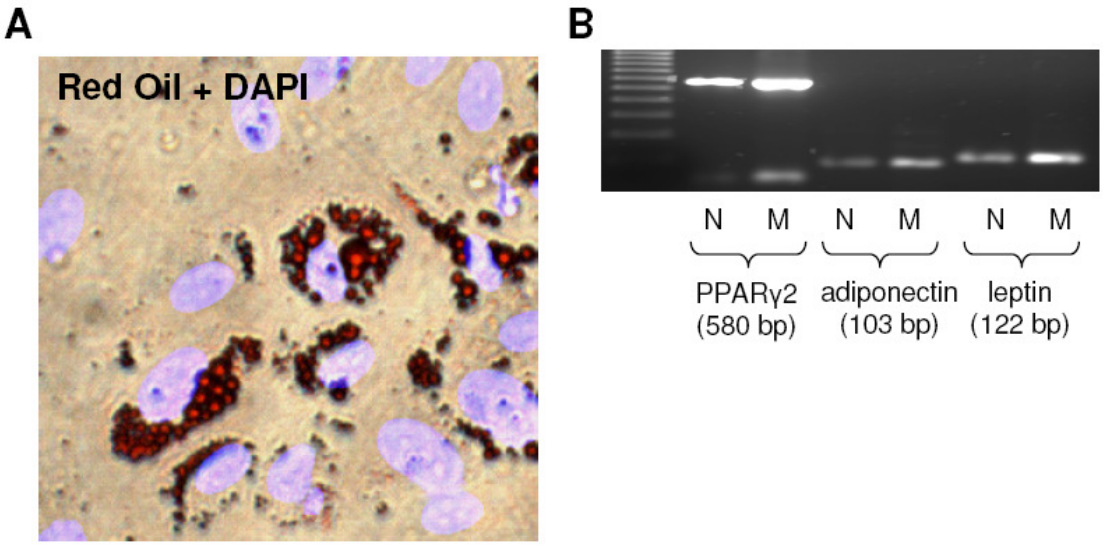

Figure 3.

Differentiation into adipocytes in vitro. A. *After culturing for 2-3 weeks in adipogenic differentiation medium, immortalized cells formed fat droplets as visualized by oil red staining. Nuclear staining was performed with DAPI. Original magnification x400.* ***B.*** *Expression of adipocyte-specific genes PPARγ2, adiponectin, and leptin in differentiated cells (N). The adipocyte-specific genes are not expressed by cultured immortalized cells (not shown). Mesenchymal stem cell derived adipocytes were used as positive control (M).*

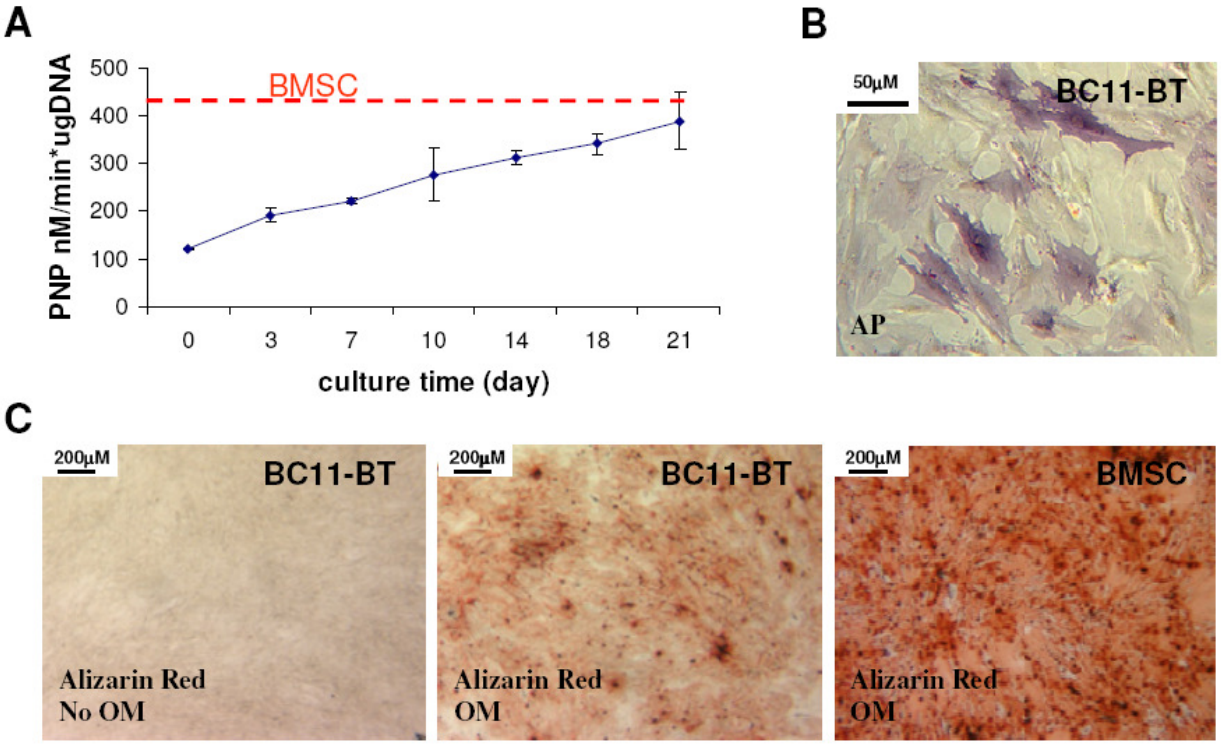

Figure 4.

Differentiation into an osteoblast-like phenotype in vitro. A*. Alkaline phosphatase (AP) activity increases during culture of immortalized cells (BC11-BT) in osteogenic medium (OM) reaching levels comparable to those expressed by bone marrow derived mesenchymal stem cells (BMSC).* ***B.*** *AP positive cells were stained in blue. Original magnification x400.* ***C.*** *BC11-BT cells formed foci of mineralization visualized by Alizarin red after 3 weeks' culture in OM. BMSC cultured in OM were used as positive control, BC11-BT cells cultured without OM were used as negative control. Original magnification x100.*

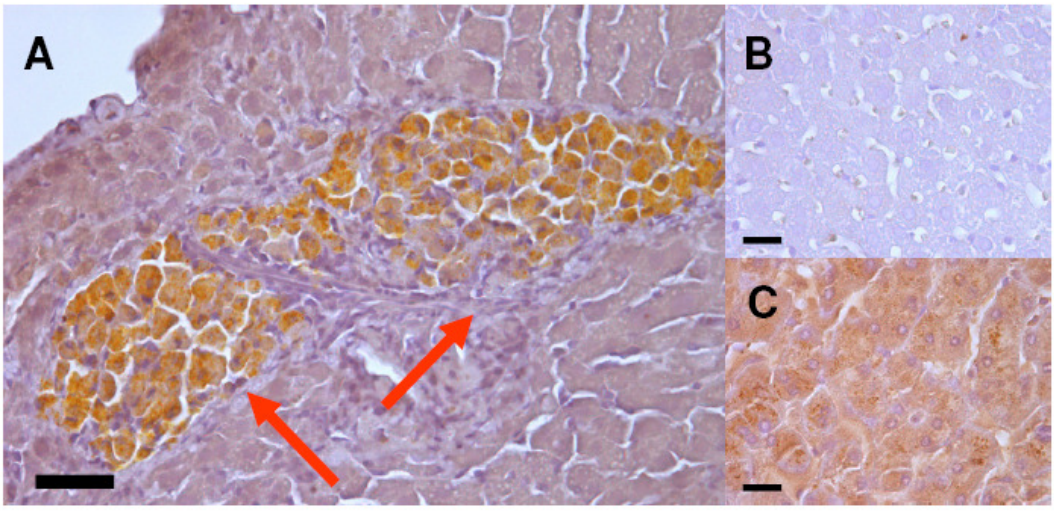

Figure 5.

Differentiation into a hepatic phenotype in vivo. *Immunohistochemistry 3 weeks after transplantation of human immortalized pancreatic cells into SCID mouse liver using a monoclonal antibody specific for human albumin and diaminobenzidine (brown) for staining* ***(A)****, nontransplanted SCID mouse liver as negative control* ***(B)****, and human liver* ***(C)*** *as positive control (bar = 20 µm).*

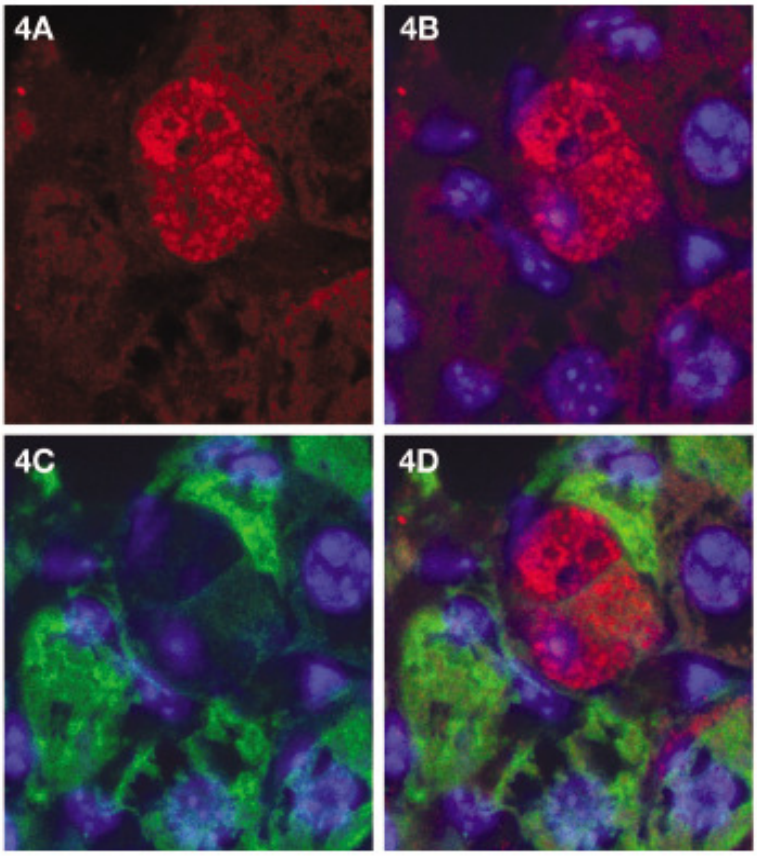

Figure 6.

Differentiation into a hepatic phenotype in vivo. *Fluorescence-immunohistochemistry with human and mouse specific antibodies against albumin using confocal microscopy (magnification x630).* ***(A):*** *One cell stained with antibodies against human albumin.* ***(B):*** *The same cell with additional 4',6'-diamidino-2-phenylindole staining for cell nuclei.* ***(C):*** *Albumin staining with antibodies against mouse albumin.* ***(D):*** *Digital overlay of human and mouse albumin staining showing no co-staining for mouse albumin in the human albumin-positive cell.*

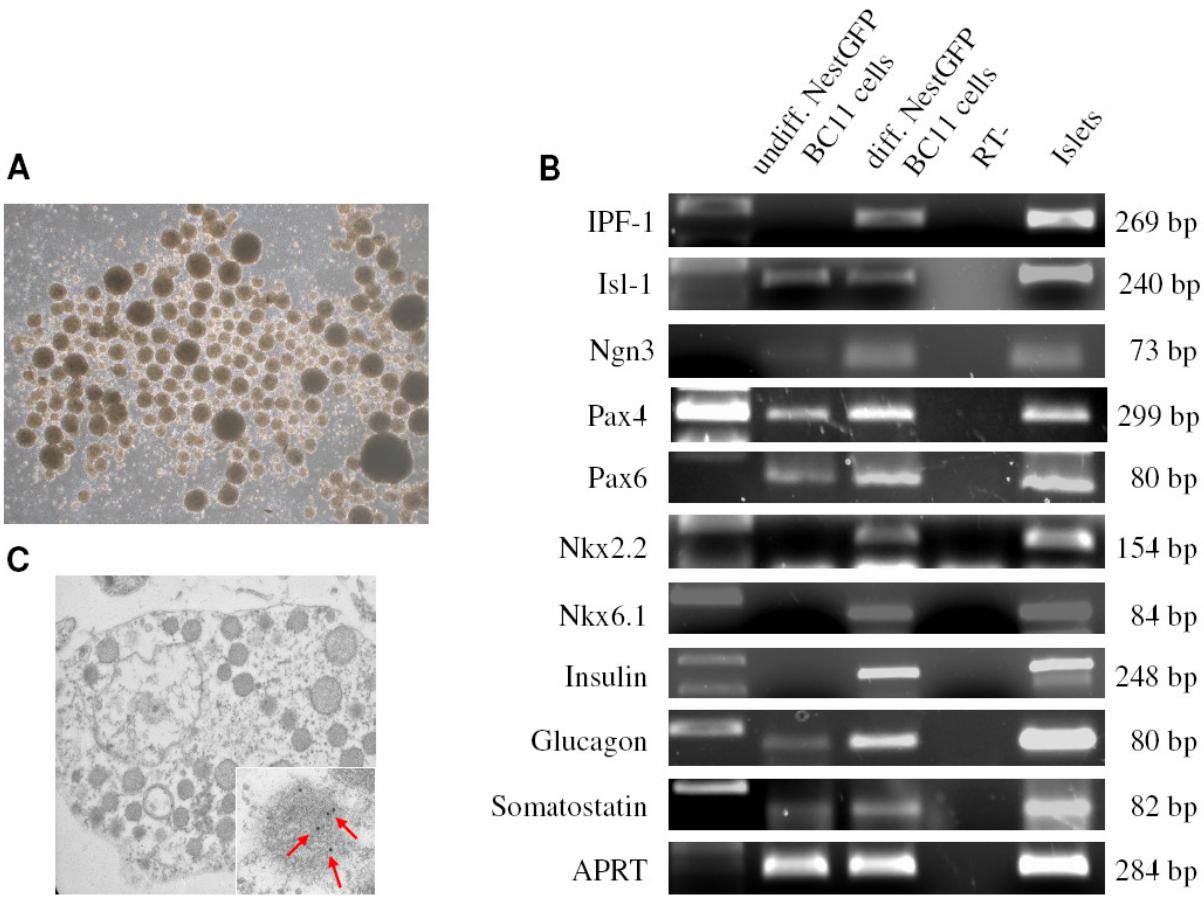

Figure 7.

Differentiation into an endocrine phenotype in vitro. ***A.*** *After 4 days of culture in differentiation medium the cells formed islet like clusters (phase contrast image).* ***B.*** *Expression of transcription factors known to play an important role in pancreatic endocrine formation including Ipf-1, Isl-1, Ngn3, Pax4, Pax6, Nkx2.2, and Nkx6.1 as well as the mRNA transcripts of the islet hormones insulin, glucagon and somatostatin.* ***C.*** *Electron microscopy studies revealed formation of granules in differentiated cells in contrast to undifferentiated cells (not shown). Red arrows indicate immunogold labeling for C-peptide (inset).*

The developmental origin of our islet derived nestin and Isl-1 positive MSC however remains somewhat elusive. They could represent indigenous stem / progenitor cells present in the adult pancreas or originate from non pancreatic tissue like bone marrow and belong to a pool of circulating stem / progenitor cells [56] that also reside in the pancreas. As well, pancreatic epithelial β-cells could de-differentiate into nestin positive cells of a mesenchymal phenotype by the mechanism of epithelial to mesenchymal transition (EMT). Epithelial cells are known to undergo EMT when placed in culture in the presence of high serum concentrations and supplemented with growth factors such as FGF2 and EGF [57-61]. Stem / progenitor cells with a mesenchymal phenotype have been described to arise in human islet cultures by EMT induced by growth factors [62]. Upon culture in serum containing medium decrease of C-peptide positive cells was observed together with concomitant increase of nestin positive cells. Besides nestin, they expressed vimentin and were able to re-differentiate into insulin expressing cells given the appropriate stimuli.

Whether EMT occurs *in vivo* in adult pancreas is unknown, but it could explain the controversial report questioned the entire concept of stem / progenitor cells in adult pancreas [63]. In this study, it has been shown that pre-existing β-cells rather than stem / progenitor cells are the major source of new β-cells concluding that adult β-cells are formed by self-duplication. The possibility of stem / progenitor cells to play a role in β-cells replacement in adult life was almost excluded. However, another interpretation of these data is that new β-cells can be generated from pre-existing β-cells by EMT indicating that this mechanism may occur *in vivo* in pancreas.

Expanding the concept of EMT, MSC from other organs like bone marrow or adipose tissue could also harbour the potential to differentiate into an endocrine pancreatic phenotype. Bone marrow derived MSC can differentiate into hepatocytes *in vitro* and *in vivo* [52,64] and hepatic progenitor cells are able to generate insulin producing cells *in vitro* [18,19]. MSC isolated from

murine and rat bone marrow were already shown to have the capacity to differentiate into insulin secreting cells *in vitro* and to reverse hyperglycaemia in an animal model of diabetes [26,65]. Furthermore, human bone marrow derived MSC were described to express at low level the islet transcription factor Nkx-6.1 and they were able to differentiate into insulin expressing cells upon adenoviral transduction with vectors encoding the transcription factors Ipf-1, Hlxb-6 or Foxa-2 [66]. Thus, the developmental potential of cultured MSC could include the differentiation in cells of endodermal origin like the liver and perhaps even the endocrine pancreas. Interestingly, MSC were also shown to bear the potential to adopt a neural phenotype *in vitro* and *in vivo* in rodents and humans [67-70] suggesting a neuro-endocrine developmental capacity of these cells.

We have isolated MSC from human bone marrow as well as from adipose tissue and observed a similar induction of pancreatic developmental transcription factors including the islet genes insulin, glucagon and somatostatin (Figures 8 and 9). Noteworthy, this was achieved without any genetic modification. Importantly, bone marrow derived MSC from patients with long standing type 1 diabetes were also induced to express insulin and glucagon in a similar way (Seboek D*, Timper K*, **Eberhardt M**, Linscheid P, Keller U, Martin I, Barbero A, Müller B and Zulewski H. *"Human mesenchymal stem cells from non-diabetic and type 1 diabetic patients differentiate into insulin, somatostatin and glucagon expressing cells"*. In preparation) and (Timper K*, Seboek D*, **Eberhardt M**, Linscheid P, Christ-Crain M, Keller U, Müller B and Zulewski H. *"Human adipose tissue-derived mesenchymal stem cells differentiate into insulin, somatostatin and glucagon expressing cells"*. **Biochem Biophys Res Commun** 2006; 341: 1135 – 1140)

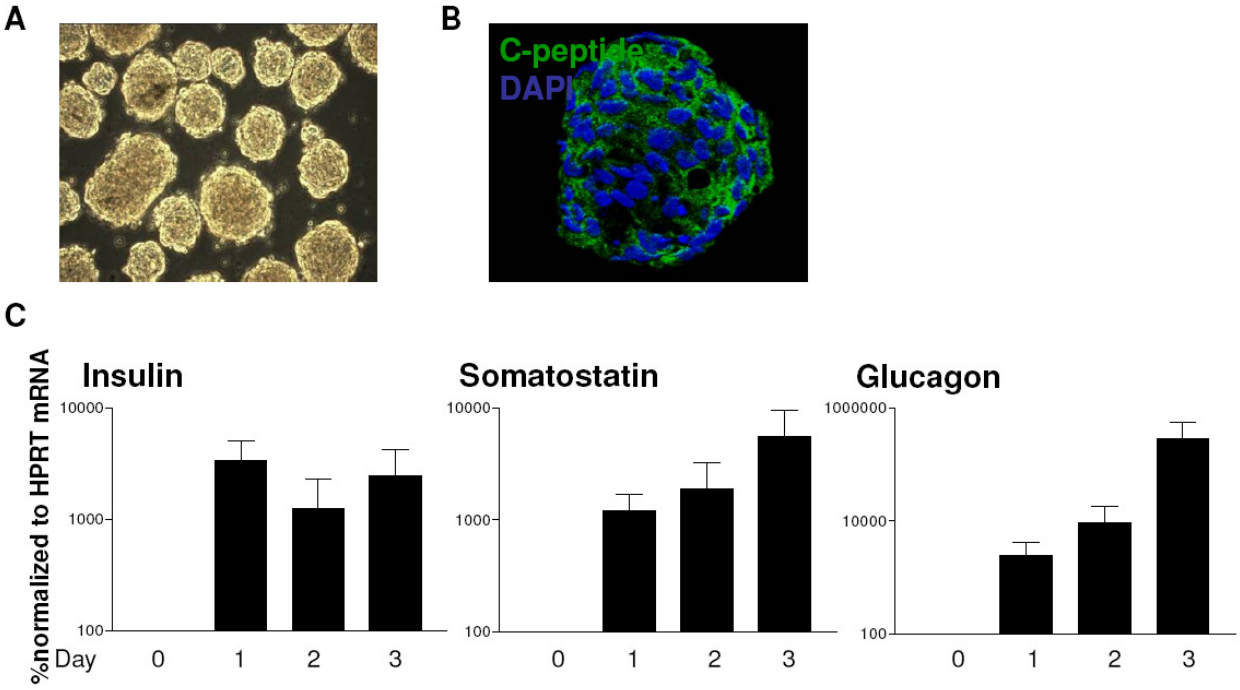

Figure 8.

Analysis of islet like clusters from bone marrow derived MSC from non-diabetic patients. ***A.*** *Phase contrast image of islet like clusters after differentiation (magnification x200).* ***B.*** *Staining of a differentiated islet like cluster for C-peptide after 3 days in differentiation medium. Nuclear staining was performed in blue with DAPI (magnification x200).* ***C.*** *Induction of insulin, somatostatin and glucagon in response to defined culture conditions. Gene expression was monitored every 24h for 3 days. Data are normalized to HPRT and expressed as percentage expression as compared to day 0. Results are means ± SEM of quadruplicates of 6 independent experiments from 5 independent donors.*

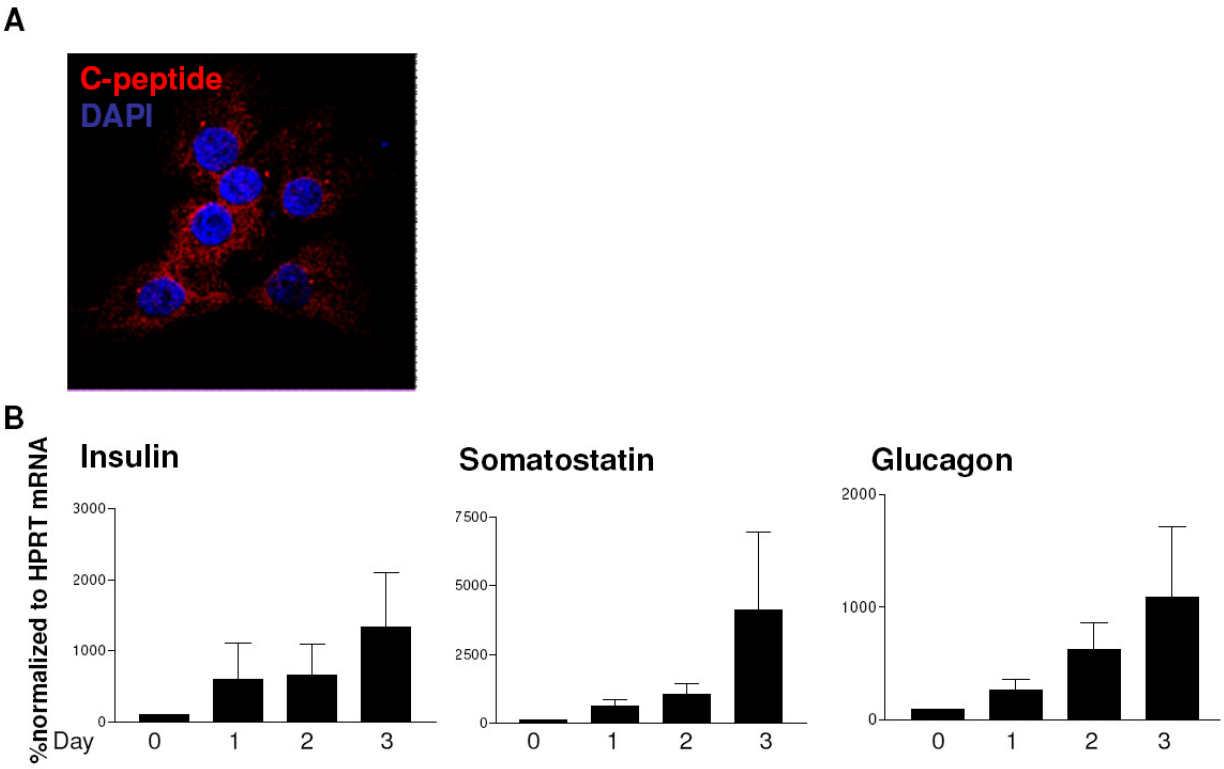

Figure 9.

Analysis of islet like clusters from adipose tissue derived MSC. A. *Immunocytochemistry for C-peptide in differentiated adipose tissue derived MSC after 3 days in differentiation medium. Single cells were stained for c-peptide (magnification x200). Nuclear staining was performed in blue with DAPI.* ***B.*** *Induction of insulin, somatostatin and glucagon in response to defined culture conditions. Gene expression was monitored every 24h for 3 days. Data are normalized to HPRT and expressed as percentage expression as compared to day 0. Results are means ± SEM of quadruplicates of 4 independent experiments from 4 independent donors.*

Although a release of somatostatin was detected, we were not yet able to measure C-peptide. But, for the first time we could show that cultured human MSC from the bone marrow as well as adipose tissue may have the potential to adopt a pancreatic endocrine phenotype without any genetic modification.

The beneficial effect of our differentiation medium was also tested in another human cell system in a collaborative project with Claes Wollheim and Benoit Gauthier (University of Geneva). Macrophage-colony stimulating factor (M-CSF) treated peripheral blood monocytes [71,72] were shown to differentiate into various cell types including insulin expressing cells. Interestingly, adenoviral-mediated forced expression of key pancreatic transcription factors like mPdx1, mNgn3 and mPax4 in M-CSF treated human monocytes did not generate insulin expressing cells. Treatment with our defined differentiation medium however resulted in a sustained up-regulation of key transcription factors as well as insulin and glucagon (data not shown).

Our cultured MSC isolated from pancreas, bone marrow and adipose tissue express all nestin but also Isl-1. The appearance of nestin and Isl-1 positive cells is most likely the result of our *in vitro* conditions. Numerous cell types start to express nestin after *in vitro* cultivation and they are believed to be stem / progenitor cells [8,10,16,67,68,73]. Nestin expression has been therefore proposed to be a property of *in vitro* multilineage stem / progenitor cells [74]. An explanation for isolation of Isl-1 positive cells from pancreatic islets could be the mechanism of EMT. Following this hypothesis endocrine cells which are positive for Isl-1 may have maintained their Isl-1 expression during the de-differentiation into MSC. If the concept of EMT holds true one could argue that Isl-1 positive MSC from other sources could also harbour the potential to differentiate into an endocrine pancreatic phenotype if exposed to appropriate culture conditions. Isl-1 expression in our bone marrow and adipose tissue derived MSC may thus represent a critical event that allowed the differentiation into an endocrine pancreatic phenotype with expression of

insulin, glucagon and somatostatin *in vitro*. But, Isl-1 is also involved in the development of the central nervous system especially motorneurons [75,76] and it has been recently also shown to be involved in the development of the heart [77,78]. Thus, at least some of our Isl-1 positive MSC may also represent potential precursors for these organs as well. Beside nestin and Isl-1 our proliferating MSC are also positive for ABCG2 which was described as a stem cell marker for the side population phenotype in bone marrow [79]. Recently ABCG2 was also found in neural stem cell and pancreatic islet derived progenitor cells [80,81]. Nestin and possibly ABCG2 expression could therefore represent a possible link between MSC and their ability to differentiate into neuro-endocrine cells.

4. OUTLOOK / FUTURE PERSPECTIVES

There is an increasing body of evidence suggesting that MSC are able to differentiate into insulin producing cells and to correct hyperglycaemia, at least in rodents [21,24,26]. Although our isolated MSC from human pancreas, bone marrow and adipose tissue have the potential to undergo differentiation into an endocrine phenotype these cells were not yet able to secrete C-peptide in response to glucose. Some of the factors needed to generate insulin expressing cells in MSC are included in our differentiation cocktail, but important elements are still lacking.

Organisation and differentiation of stem / progenitor cells during development occur in a well defined spatial and temporal context. Signals involved in cell fate decisions like SHH exert their effect also as gradient morphogens [82]. This concept of development may also apply to adult stem / progenitor cells. During development SHH signalling inhibits pancreas formation and differentiation of endocrine cells in embryonic tissue explants whereas suppression of SHH signalling with cyclopamine promotes pancreas formation [83]. Interestingly, SHH signalling pathway is known to be involved in cell proliferation and cyclopamine was shown to suppress the expression of nestin as well as Bmi-1 that is needed for self renewal of adult stem cells [84,85]. Our preliminary data show that genes required for transduction of hedgehog signals like Gli1, Patched and smoothened are expressed in proliferating bone marrow derived MSC (Figure 10A). Interestingly, when they were analysed before and after induction of differentiation we observed a down-regulation of the hedgehog target gene Gli1 that represents a direct readout of the hedgehog signalling pathway (Figure 10B). In addition, treatment with 10μM cyclopamine for 72 hours was associated with an increase in Isl-1 expression (Figure 10C). This first pilot experiment indicates that SHH signalling may play an important role in

proliferation and differentiation of bone marrow derived MSC. A pre-incubation with cyclopamine could therefore help to improve their differentiation efficacy. A prolonged culture for additional days after induction of differentiation may also enhance the maturation of islet like clusters.

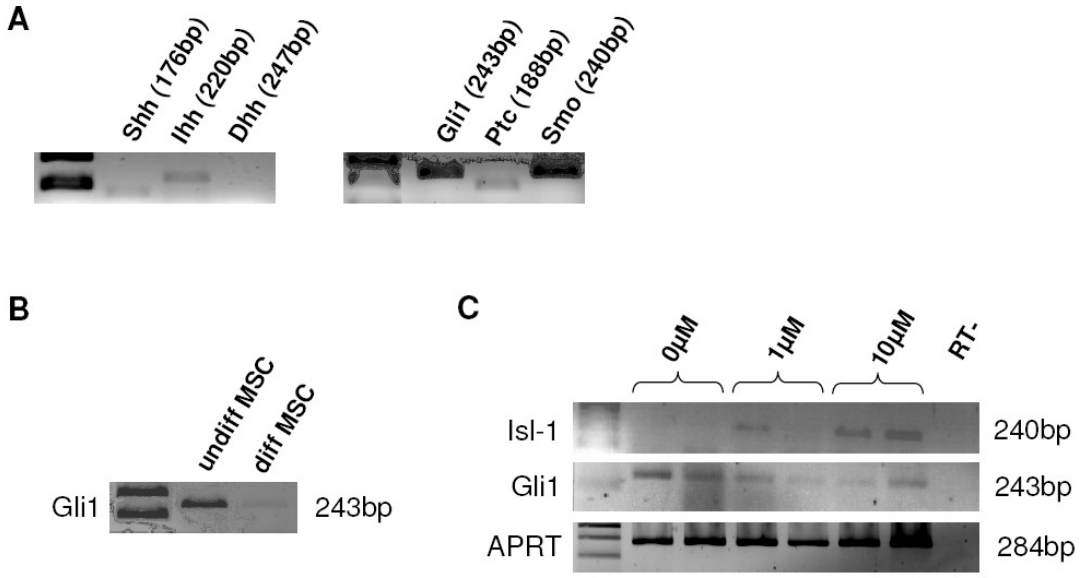

Figure 10.

Sonic hedgehog signaling pathway is involved in human bone marrow derived mesenchymal stem cells (bMSC) A. *RT-PCR analysis showed that mRNA of Sonic (Shh) and Indian (Ihh) hedgehog as well as target genes of the Shh signaling pathway including Gli1, Patched (Ptc) and Smoothened (Smo) are expressed in bMSC whereas Desert (Dhh) hedgehog is not found.* ***B.*** *Down-regulation of Gli1 mRNA of human bMSC after differentiation for 3 days into cells with pancreatic endocrine phenotype.* ***C.*** *Human bMSC were treated with cyclopamine (0µM, 1µM and 10µM) for 72 h and analyzed by RT-PCR. SHH target gene Gli1 is down-regulated whereas the crucial pancreatic endocrine transcription factor Isl-1 is up-regulated. Adenine phosphoribosyltransferase (APRT) was used as positive control for RT-PCR.*

In addition, MSC derived insulin expressing islet like clusters have to be further characterized regarding their functional properties. This includes analysis of glucose dependent C-peptide release as well as their potential to correct hyperglycaemia in an animal model of diabetes. Islet like clusters have been shown to undergo a significant maturation after transplantation *in vivo* and to reverse diabetes in an animal model [15,16,24].

Using our differentiation protocol the formation of islet like clusters is difficult to control. The floating clusters in the ultra-low attachment tissue culture dishes differ considerably in size. Too many cells in one cluster may lead to apoptosis in the centre of the cluster because of reduced access for oxygen and nutrients. But it is likely that also local factors secreted within these clusters are crucial for differentiation. Thus, a critical cell size may be required and has to be defined for optimal differentiation of MSC into insulin producing cells. The optimal numbers of MSC for best insulin expression could be evaluated using the hanging drop technique that allows the deposition of a defined cell number in a defined volume of differentiation medium.

In conclusion, the work presented herein demonstrate for the first time that human MSC from various tissues are able to induce pancreatic endocrine genes including crucial transcription factors as well as glucagon, somatostatin and insulin. Further studies are required to enhance the differentiation efficacy.

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6. PAPERS

The results of this thesis are based on the following publications / manuscript. Asterics (*) indicate equal contributions by the authors.

6.1 **Eberhardt M***, Salmon P*, von Mach MA, Hengstler JG, Brulport M, Linscheid P, Seboek D, Oberholzer J, Barbero A, Martin I, Müller B, Trono D and Zulewski H. *"Multipotential nestin and Isl-1 positive mesenchymal stem cells isolated from human pancreatic islets"*. Biochem Biophys Res Commun 2006; 345: 1167 – 1178.

6.2 von Mach MA, Hengstler JG, Marc Brulport M, **Eberhardt M**, Schormann W, Hermes M, Prawitt D, Zabel B, Grosche J, Reichenbach A, Müller B, Weilemann LS and Zulewski H. *"In vitro cultured islet-derived progenitor cells of human origin express human albumin in SCID mouse liver in vivo"*. Stem Cells 2004; 22: 1134 – 1141.

6.3 Seboek D*, Timper K*, **Eberhardt M**, Linscheid P, Keller U, Martin I, Barbero A, Müller B and Zulewski H. *"Human mesenchymal stem cells from non-diabetic and type 1 diabetic patients differentiate into insulin, somatostatin and glucagon expressing cells"*. In preparation.

6.4 Timper K*, Seboek D*, **Eberhardt M**, Linscheid P, Christ-Crain M, Keller U, Müller B and Zulewski H. *"Human adipose tissue-derived mesenchymal stem cells differentiate into insulin, somatostatin and glucagon expressing cells"*. Biochem Biophys Res Commun 2006; 341: 1135 – 1140.

6.1

Multipotential nestin and islet-1 positive mesenchymal stem cells isolated from human pancreatic islets

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Multipotential nestin and Isl-1 positive mesenchymal stem cells isolated from human pancreatic islets

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Abstract

Mesenchymal cells in the developing pancreas express the neural stem cell marker nestin and the transcription factor islet-1 (Isl-1). Using defined culture conditions we isolated on a single cell basis nestin producing cells from human pancreatic islets. These cells were immortalized with lentiviral vectors coding for telomerase and mBmi. They are positive for Isl-1 and nestin and have the potential to adopt a pancreatic endocrine phenotype with expression of critical transcription factors including Ipf-1, Isl-1, Ngn-3, Pax4, Pax6, Nkx2.2, and Nkx6.1 as well as the islet hormones insulin, glucagon, and somatostatin. In addition, they can be differentiated into human albumin producing cells in vivo when grafted into a SCID mouse liver. In accordance with a mesenchymal phenotype, the cells were also able to adopt adipocytic or osteocytic phenotypes in vitro. In conclusion, cultured pancreatic islets contain nestin and Isl-1 positive mesenchymal stem cells with multipotential developmental capacity.

Keywords: Human; Pancreatic islet; Nestin; Mesenchymal stem cells; ABCG2; Isl-1; Differentiation; Diabetes; Insulin; Albumin

The development of normal pancreas is the result of close interaction between mesenchymal and epithelial cells that form the initial buds. Signals from mesenchymal cells direct pancreatic development towards endocrine or exocrine fate [1]. Mesenchymal cells of the developing pancreas express islet 1 (Isl-1) [2], an essential transcriptional factor for the generation of endocrine cells and nestin [3], a neural stem cell marker [4] that was also identified within adult pancreatic islets [5]. Fate and function of pancreatic mesenchymal cells in postnatal life are unknown. They could represent a population of dormant mesenchymal stem cells (MSC) with the potential to differentiate into pancreatic endocrine or even hepatic phenotype given the appropriate stimuli [5]. Accordingly in recent in vitro

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studies nestin positive cells isolated from fetal human pancreas could be differentiated into insulin producing cells and cured diabetes in an animal model [6,7].

Here we demonstrate that multipotent mesenchymal cells can be found in primary cultures of human islets. We further describe the isolation and immortalization of single cell derived nestin and Isl-1 positive cells from adult human islets of Langerhans. These cells display a mesenchymal phenotype and can be induced to differentiate towards a pancreatic endocrine, an adipocytic or an osteocytic phenotype in vitro and a hepatic phenotype in vivo.

Research design and methods

Cell culture studies. Human islet tissue was obtained from the Islet Transplantation Center in Geneva University Hospital, (Geneva Switzerland). Nestin positive islet derived progenitor cells were isolated as described previously [5]. Briefly, islets were washed and cultured in expansion medium (RPMI 1640 medium containing 10% fetal bovine serum, antibiotics, sodium pyruvate (1 mM), Hepes (10 mM), and β-mercaptoethanol (50 mM)). Within several days, nestin positive cells grew out from islets. These cells were expanded in medium containing 20 ng/ml each of FGF2 and EGF. After two passages, cells were re-suspended in a single cell suspension and subjected to a single cell deposition treatment by a FACS (Becton–Dickinson, Palo Alto, CA). EGF was from Sigma (Buchs, Switzerland). All other reagents were from Invitrogen/Gibco BRL (Basel, Switzerland).

Cell transduction using lentiviral vectors. HLOX vectors used for immortalization (pLOX-CW-Bmi1 and pLOX-TERT-iresTK) have been described previously [8,9], and express the murine Bmi-1 cDNA [10] (GenBank No. M64067) and the human hTERT cDNA (GenBank No. AF018167) followed by the thymidine kinase of Herpes simplex virus type 1 (HSV1-TK), respectively, in a pLox backbone. The vector used for transcription activated cell sorting (TRACS) was constructed as follows. Briefly, the neuron-specific GFP transgene described by Yamaguchi et al. [11] and comprising the rat nestin promoter, the EGFP cDNA (Clontech), the neuron-specific second intron of the rat nestin gene, and the chicken β-globin polyadenylation signal was inserted in opposite orientation into a CCL-LoxP HIV-1 derived vector. Detailed maps and sequences of the lentiviral vectors used in this study can be obtained at http://www.medecine.unige.ch/~salmon.

Production of HIV derived vectors pseudotyped with the VSV G envelope protein was achieved by transient co-transfection of three plasmids into 293T epithelial cell line as previously described [12]. Titers of LV stocks were determined by transduction of target cells and measuring of the fraction of transgene-expressing cells by flow cytometry (for GFP-expressing vectors) or number of integrated proviral genomes by quantitative PCR (for Bmi-1 or hTERT-expressing vectors). Details on titration techniques are available at http://www.medecine.unige.ch/~salmon. The functionality of the nestin-GFP LV was assessed by transduction of the nestin-expressing NFSK-1 human neuronal cell line (ATCC CRL-2060). The tissue-specificity of the nestin-GFP cassette in the context of the lentiviral vector was confirmed by GFP expression in NFSK cells and absence of GFP expression in HeLa cells (data not shown). Using a ubiquitous promoter (PGK or EF1), NFSK and HeLa cells were shown to be equally transduceable by LVs (data not shown). Titers of nestin-GFP LV stocks as determined by FACS on NFSK cells were thus considered equivalent to titers determined on HeLa cells. BC11 cells were transduced by lentiviral vectors at multiplicities of infection (MOI) ranging from 0.5 to 2.

Reverse transcription and polymerase chain reaction. Total RNA from human islets, cell cultures, and liver samples was extracted with TRIzol reagent (Lucerna Chemie AG, Switzerland). RNA (1 μg) was then reverse transcribed using Omniscript (Qiagen, Basel, Switzerland) and amplified by polymerase chain reaction (PCR) using *Taq* PCR core kit (Qiagen). Negative controls without reverse transcriptase enzyme were run in parallel to exclude possible contamination. Human gene-specific, intron spanning primers are listed in Table 1. PCR products were separated and visualized on agarose gels containing 0.5 μg/ml ethidium bromide (EtBr, Bio-Rad Laboratories AG, Reinach, Switzerland). One hundred bp molecular ruler (Bio-Rad) was run as size reference. PCR product identity was confirmed by nucleotide sequencing (Microsynth AG, Balgach, Switzerland).

Differentiation of immortalized BC11 cells. For differentiation into an endocrine phenotype, 90% confluent BC11 cells were trypsinized and seeded in low attachment plates (Vitaris, Baar, Switzerland) containing serum-free DMEM-F12 medium (17.5 mM) supplemented with B-27

Table 1
Primer sequences for RT-PCR

Gene	Sense primer	Antisense primer	Amplicon (bp)	Accession No.	Cycles
Nestin	5′-CGTTGGAACAGAGGTTGGAG-3′	5′-TAAGAAAGGCTGGCACAGGT-3′	396	BC032580	40
ABCG2	5′-CACAGGTGGAGGCAAATCTT-3′	5′-TCCAGACACACCACGGATAA-3′	322	AY289766	40
Thy-1	5′-GTCCTTTCTCCCCCAATCTC-3′	5′-GGGAGACCTGCAAGACTGTT-3′	239	NM_033209	40
SCF	5′-GGTGGCAAATCTTCCAAAAG-3′	5′-TCTTTCACGCACTCCACAAG-3′	222	BC074725	40
c-Kit	5′-GGCATCACGGTGACTTCAAT-3′	5′-GGTTTGGGGAATGCTTCATA-3′	244	L04143	40
Isl-1	5′-GTTACCAGCCACCTTGGAAA-3′	5′-TTCCCACTTTCTCCAACAGG-3′	240	BC031213	40
Insulin	5′-GGCTTCTTCTACACACCCAAG-3′	5′-CATCTCTCTCGGTGCAGGA-3′	248	AY138590	40
Glucagon	5′-CCCAAGATTTTGTGCAGTGGTT-3′	5′-CAGCATGTCTCTCAAATTCATCGT-3′	80	NM_002054	40
Somatostatin	5′-GATGCCCTGGAACCTGAAGA-3′	5′-CCGGGTTTGAGTTAGCAGATCT-3′	82	BC032625	40
Ipf-1	5′-CCTTTCCCATGGATGAAGTC-3′	5′-TTGTCCTCCTCCTTTTTCCA-3′	269	AF035259	40
Ngn-3	5′-CTATTCTTTTGCGCCGGTAGA-3′	5′-CTCACGGGTCACTTGGACAGT-3′	73	NM_020999	40
Pax-4	5′-TCTCCTCCATCAACCGAGTC-3′	5′-GTTGGAAAACCAGACCCTCA-3′	299	AF043978	40
Pax-6	5′-TGCGACATTTCCCGAATTCT-3′	5′-GATGGAGCCAGTCTCGTAATACCT-3′	81	NM_001604	40
Nkx2.2	5′-TCTACGACAGCAGCGACAAC-3′	5′-TTGTCATTGTCCGGTGACTC-3′	154	O95096	40
Nkx6.1	5′-TCTTCTGGCCCGGAGTGA-3′	5′-CCAACAAAATGGATCCTTGATGA-3′	84	NM_006168	40
Albumin	5′-ACTTTTATGCCCCGGAACTC-3′	5′-AGCAGCAGCACGACAGAGTA-3′	598	NM_000477	40
PPARγ2	5′-GCGATTCCTTCACTGATAC-3′	5′-GCATTATGAGACATCCCCAC-3′	580	NM_015869	40
Leptin	5′-TGCCCATCCAAAAAGTCCA-3′	5′-GAAGTCCAAACCGGTGACTTTCT-3′	122	NM_000230	40
Adiponectin	5′-TGGGCCATCTCCTCCTCA-3′	5′-AATAGCAGTAGAACAGCTCCCAGC-3′	103	NM_004797	40
APRT	5′-GCGTGGTATTCAGGGACATC-3′	5′-CAGGGCGTCTTTCTGAATCT-3′	284	NM_000485	28

All primers were run at 60 °C.

supplement, N-2 supplement, activin-A (2 nM), nicotinamide (10 mM), exendin-4 (10 nM), hepatocyte growth factor (100 pM), and pentagastrin (10 nM). After 4 days, cells were collected for further experiments and analysis. Culture medium, B-27 supplement, and N-2 supplement were obtained from Invitrogen/Gibco BRL (Basel, Switzerland). All other reagents were from Sigma (Buchs, Switzerland) except for pentagastrin that was from Cambridge Laboratories, Newcastle Upon Tyne, UK. Differentiation into an adipocyte or osteoblastic phenotype was performed with the same culture conditions normally applied for adipocytic or osteoblastic differentiation of bone marrow derived mesenchymal stem cells (BMSC) or preadipocytes [13,14]. For these studies BMSC have been used as control cells.

In vivo investigations. 300.000 BC11 cells were transplanted into the livers of four SCID mice as described previously [15]. After 35 days, the injected liver lobes were excised and shock frozen in liquid nitrogen. RT-PCR analysis was performed for human albumin as described above. For immunohistochemistry the specimens were transferred to 4% paraformaldehyde and embedded into paraffin. Peroxidase was blocked in 7.5% H_2O_2 in methanol for 60 min at 4 °C. Unspecific binding-sites were blocked in 3% BSA (bovine serum albumin) for 2.5 h at 37 °C. Afterwards, an avidin/biotin-block (Vector Laboratories, Inc., Burlingame, CA) was performed as described by the manufacturer. Slides were then incubated with a 1:50 dilution of an affinity purified human albumin antibody produced in goat (Bethyl Laboratories, Inc., Montgomery, TX; Cat. No. A80-229A) for 60 min at rt. Detection of the first antibody was performed using commercial Vectastain Elite ABC Kit (Vector Laboratories) as described by the manufacturer, followed by a 5 min incubation with 0.6 mg/mL diaminobenzidine at room temperature. Finally the sections were counterstained with a 1:5 dilution of Mayer's hemalum (Merck, Darmstadt, Germany).

Histochemical stainings. Differentiated cells were rinsed off with PBS and fixed in 4% formaldehyde for 30 min. Standard Oil Red O, alkaline phosphatase, and Alizarin Red staining were performed as previously described [16].

Immunocytochemistry. Expanded cells were transferred into Lab-Tek chamber slides (Nunc, Naperville, IL) and incubated overnight in expansion medium. Islet-like clusters were collected after 4 days in differentiation medium, transferred onto glass slides coated with poly-L-lysine (Sigma), and incubated overnight in DMEM-F12 medium containing 10% FCS. All cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 30 min at rt. After several rinses in PBS, cells were permeabilized with chilled methanol for 10 min. To prevent unspecific bindings they were then incubated with 10% fetal bovine serum in PBS at rt for 30 min. Expanded cells were incubated with primary antisera for 3 h at rt or overnight at 4 °C, rinsed off with PBS, and incubated with secondary antisera for 1 h at rt. Islet-like clusters were incubated with primary antisera for 60–90 min at 37 °C, rinsed off with PBS, and incubated with secondary goat antisera for 45 min at 37 °C. After several washes with PBS, they were finally coverslipped with non-fluorescing mounting medium. The primary antibodies used were rabbit anti-nestin (dilution 1:200) (Chemicon, Juro Supply, Lucerne, Switzerland), rabbit anti-human c-peptide (dilution 1:1000) (Linco, St. Charles, MO), and rabbit anti-glucagon (dilution 1:100) (Linco, St. Charles, MO). Mouse anti Isl-1 39.4D5 (1:100 dilution) was obtained from the Developmental Studies Hybridoma Bank, University of Iowa, USA. The secondary antibodies from Molecular Probes (Invitrogen/Gibco BRL) were goat anti-mouse (dilution 1:1000) and goat anti-rabbit (dilution 1:1000). Goat anti-mouse IgG was labeled with Alexa Fluor 488 dye and goat anti-rabbit IgG was labeled with Alexa Fluor 546 dye. 4,6-Diamidino-2-phenylindole (DAPI, 5 μg/ml) from Sigma was used to label the nuclei for 10 min at 37 °C. Cells were then examined either by fluorescence microscope (Axiophot, Zeiss, Germany) or by confocal microscope (LSM 510, Zeiss, Germany). The insulinoma cell line INS IE was provided by C. Wollheim, Geneva, Switzerland. HeLa cells were from ATCC (Manassas, VA).

Electron microscopy with immunogold labeling. Cells were fixed in a mixture of 3% paraformaldehyde and 0.5% glutaraldehyde in 10 mM PBS, pH 7.4, for 1 h. Cells were then washed in PBS and fixed in 0.5% osmiumtetroxide for 30 min. After rinsing with water, cells were dehydrated in ethanol and embedded in LR White. Sixty nanometer-sections were treated with 2% BSA in PBS to block non-specific bindings and incubated with primary antibody in 2% BSA in PBS for 2 h. After rinsing with PBS, the sections were incubated for 1 h with goat anti-rabbit IgG labeled with 10 nm gold particles (Amersham, Netherlands). The sections were washed with PBS and water, stained with uranyl acetate and lead acetate and observed and photographed in a LEO 910 TEM (Zeiss).

Flow cytometry analysis. Single cell suspension of undifferentiated and differentiated BC11-BT NestGFP cells were sorted with FACScan (Becton–Dickinson, Palo Alto, CA) based on GFP fluorescence. BC11-BT cells were used as standard for cell size and aspect, and as negative control.

Results and discussion

Isolation of single cell derived colonies

After few days of culture of purified human islets an outgrowth of cells with a fibroblast-like appearance was observed. This was paralleled by the induction of several stem cell markers including nestin, the side population stem cell marker ABCG2, stem cell factor (SCF), c-Kit, and Thy-1, a potential marker for liver stem cells [17]. During the proliferation period, the levels of insulin and Ipf-1 mRNA expression gradually decreased, to become undetectable at passage 7 (Fig. 1A). The transcription factor Ipf-1 is crucial for initiation of pancreas development and an important regulator of insulin transcription in adult life [1]. Immunocytochemistry studies (ICC) showed that almost all cells were positive for nestin (Fig. 1B). Assuming that some of these cells may represent stem cells we subjected the population (at passage 2) to single cell cloning by FACS (Fig. 1C). Two weeks later, growing colonies were observed in five of a total of 192 wells. All five colonies continued to express ABCG2 and nestin but neither insulin nor Ipf-1 (Fig. 2A). Interestingly, all colonies expressed the transcription factor Isl-1 suggestive of mesenchymal stem/progenitor cells (Fig. 2A). During development, both nestin and Isl-1 are found in pancreatic mesenchyme surrounding the epithelial cells that also express Isl-1 [2,3]. Isl-1 is well known for its critical role in the development of pancreatic endocrine cells [2].

Immortalization of single cell derived colonies

Extensive functional characterization of progenitor/stem cells requires stable and robust proliferation. For this purpose, we immortalized the best growing clone (BC11) by overexpressing telomerase alone or together with mBmi via Lox-CRE excisable lentiviral vectors as described previously [8] (Fig. 2B–D). This combination of genes has been shown to be optimal for immortalization of primary human cells without genetic alterations [9]. Furthermore, Bmi-1 was recently shown not only to promote cell cycling but also to be crucial for the maintenance of the precursor status of neuronal progenitors [18]. In an additional purification step these cells were transduced with a lentivector containing a nestin-GFP promoter construct. Cells with the highest GFP levels were then selected based on the

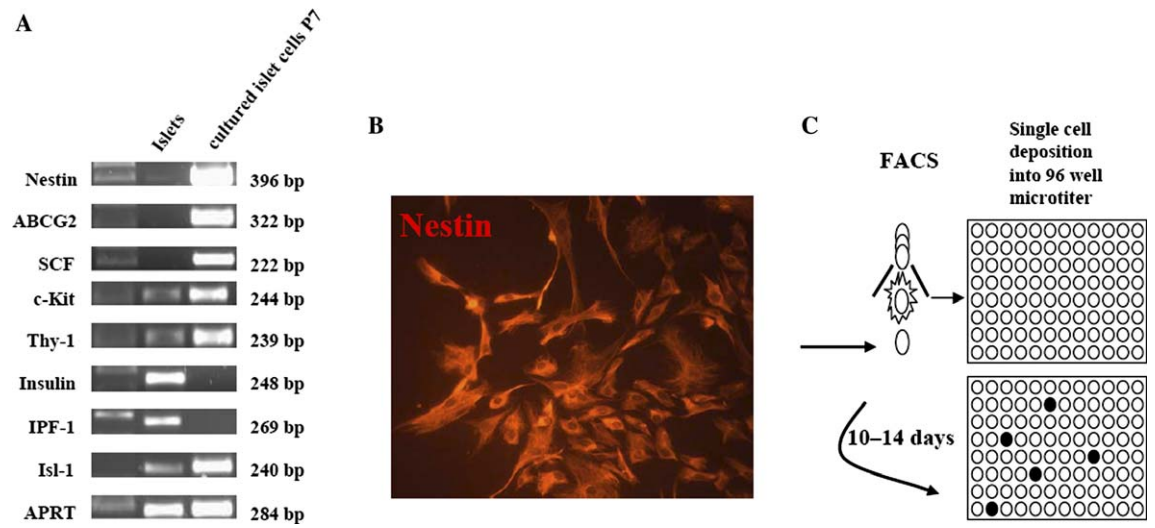

Fig. 1. (A) Purified human islets express nestin, c-Kit, Thy-1 as well as the transcription factors Isl-1, Ipf-1, and also insulin but no ABCG2 and no SCF. Cultured islets cells (passage 7) express nestin, ABCG2, Thy-1 as well as Isl-1 but no Ipf-1 and no insulin. (B) By immunocytochemistry more than 90% of cells outgrowing from the islets at passage 2 were positive for nestin. Original magnification 200×. (C) Isolation of single cell derived colonies from cultured human islet cells (passage 2) using a FACS procedure. After 10–14 days formation of proliferating colonies was observed in five of a total of 192 wells.

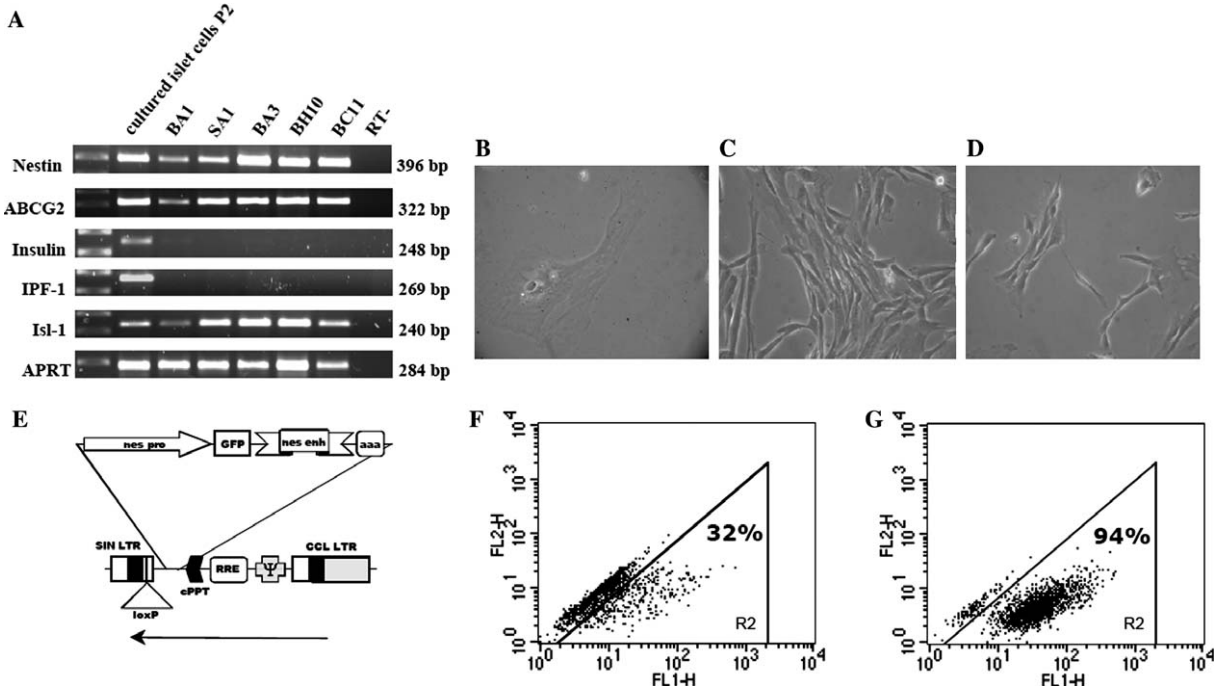

Fig. 2. (A) Expression of mRNA for nestin, ABCG2, Isl-1, insulin, and IPF-1 in cultured human islets of Langerhans (passage 2) and single cell derived colonies (BA1, SA1, BA3, BH10, and BC11). APRT mRNA expression served as positive control. The cells of origin as well as all colonies isolated expressed nestin, ABCG2, and Isl-1 but only the original cell population was positive for insulin and IPF-1. Cultured islet cells are FGF2 and EGF stimulated cells. (B–G) Clone BC11 was transduced with immortalizing HLOX lentiviral vectors and analyzed for proliferation and morphology. Passage numbers and cell counts corresponding to phase contrast analysis were as follows: (B) untransduced cells, passage 4, 5.9×10^3 cells. (C) Cells transduced with hTERT, passage 5, 2.3×10^4 cells; (D) cells transduced with mBmi-1 and hTERT, passage 5, 1.1×10^6 cells. Original magnification 200×. The BC11 cells immortalized with mBmi-1 and hTERT (BC11-BT) were then transduced with a lentiviral vector containing a tissue-specific nestin-GFP cassette depicted in (E). (F) FACS analysis of immortalized BC11-BT after transduction with CX-NestGFP vector. Cells were analyzed by flow cytometry using two channels: FL-1, green fluorescence for GFP signal, *x*-axis, 4-decade log scale; FL-2, red fluorescence for non-specific auto-fluorescence, *y*-axis, 4-decade log scale. Cells were gated for specific GFP signal (32% of viable cells in gate R2) and positively sorted for further culture. (G) GFP expression of sorted nestin-GFP positive immortalized BC11-BT NestGFP cells. Sorted cells from (F) gate R2 were analyzed as in (F) after 3 weeks of culture and expansion. The percentage indicates the fraction of cells in gate R2 (94% of viable cells in gate R2). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

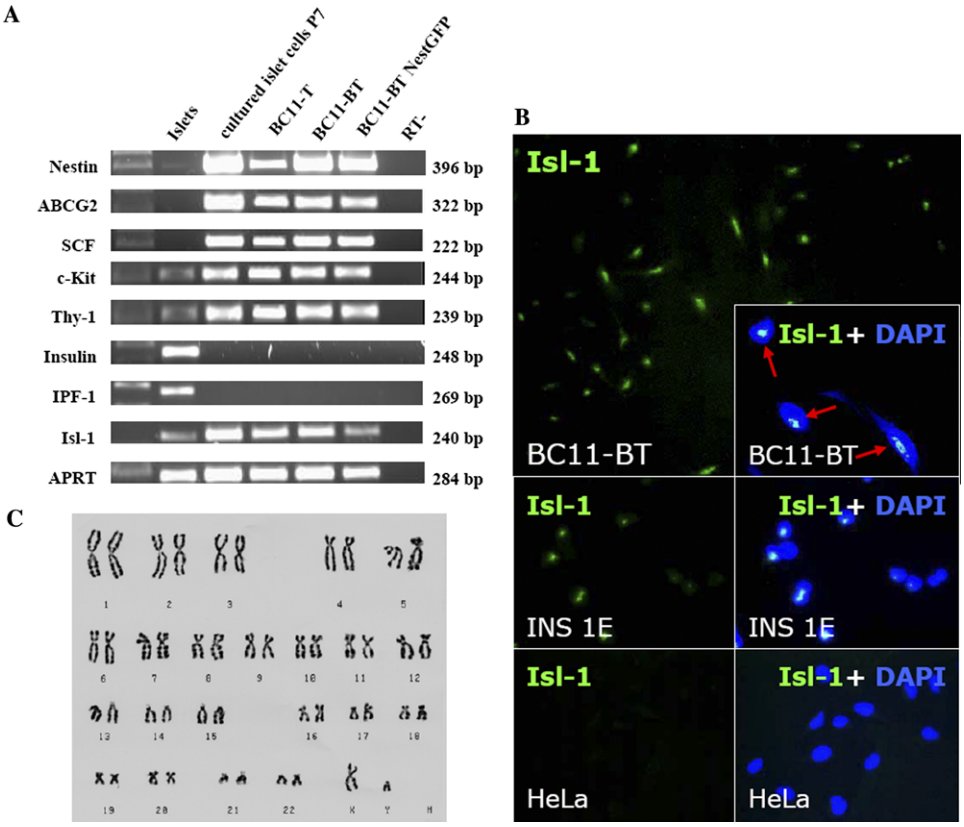

Fig. 3. (A) Expression of mRNA for nestin, ABCG2, stem cell factor (SCF), c-Kit, and Thy-1 together with Isl-1 in all immortalized single cell derived cell lines. Cultured islet cells passage 7, BC11-T denotes single cell derived cells immortalized by induction of telomerase only; BC11-BT immortalized by induction of telomerase and mBmi; BC11-BT NestGFP indicates BC11-BT additionally purified for nestin-expressing cells. (B) Immunocytochemistry for Isl-1 expression shown in immortalized BC11-BT cells. Note the nuclear staining for the transcription factor Isl-1 in immortalized BC11 cells as well as in the rat insulinoma cell line INS 1E that served as positive control. HeLa cells were not Isl-1 positive. Nuclear staining was performed with DAPI. Original magnification 200×. (C) Immortalized cell lines (up to 75 passages) have maintained their phenotype without evidence for chromosomal aberrations.

assumption that a strong nestin promoter activity correlated with enhanced stem cell potential (Fig. 2E–G). The resulting immortalized clones continued to express the stem cell markers nestin, ABCG2, SCF, c-Kit, and Thy-1 and the transcription factor Isl-1 (Fig. 3A), the latter detected in almost all cells by ICC (Fig. 3B). These immortalized cells have been cultured for up to 75 passages while maintaining their phenotype without evidence for chromosomal aberrations (Fig. 3C).

Differentiation of immortalized cells into pancreatic endocrine phenotype

To induce differentiation into a pancreatic endocrine phenotype cells were cultured in serum-free DMEM/F12 medium supplemented with factors known for their beneficial effects on differentiation of precursors into insulin producing cells such as exendin-4, nicotinamide, hepatocyte growth factor, activin-A, and pentagastrin [19–22]. During the 4 days of incubation in ultra-low attachment tissue culture dishes, we observed the formation of islet-like clusters (Fig. 4A) and the induction of transcription factors known for their pivotal role during development of pancreatic endocrine cells including Isl-1, Ipf-1, Ngn3, Pax4, Pax6, Nkx2.2, and Nkx6.1 (Fig. 4B). In addition, insulin, glucagon and somatostatin mRNAs were up-regulated (Fig. 4B) while GFP expression decreased in parallel in the NestGFP-transduced BC11-cells (Fig. 4C–E). All immortalized cell lines gave very similar results regarding their differentiation potential. Interestingly, Pax-4, Pax-6, glucagon, and somatostatin were already expressed, albeit at a lower level, before induction of differentiation. Using confocal microscopy we could identify C-peptide positive cells in some areas of differentiated islet-like clusters (Fig. 5A). C-peptide is part of the proinsulin peptide and thus an indicator of de novo proinsulin production. Glucagon positive cells were also identified at the periphery of islet-like cluster (Fig. 5A). Electron microscopy combined with immunogold staining revealed C-peptide positive granula in differentiated cells (Fig. 5B). These data are in agreement with previous reports showing the generation of insulin producing cells from cultured nestin positive cells from fetal human pancreas [6,7]. Our results however contrast with studies in which purified nestin-expressing cells isolated from human fetal pancreas were not able to adopt a pancreatic endocrine phenotype in vitro or in vivo [23]. Beside the differences in cell type, fetal pancreatic versus immortalized adult islet derived cells, this apparent

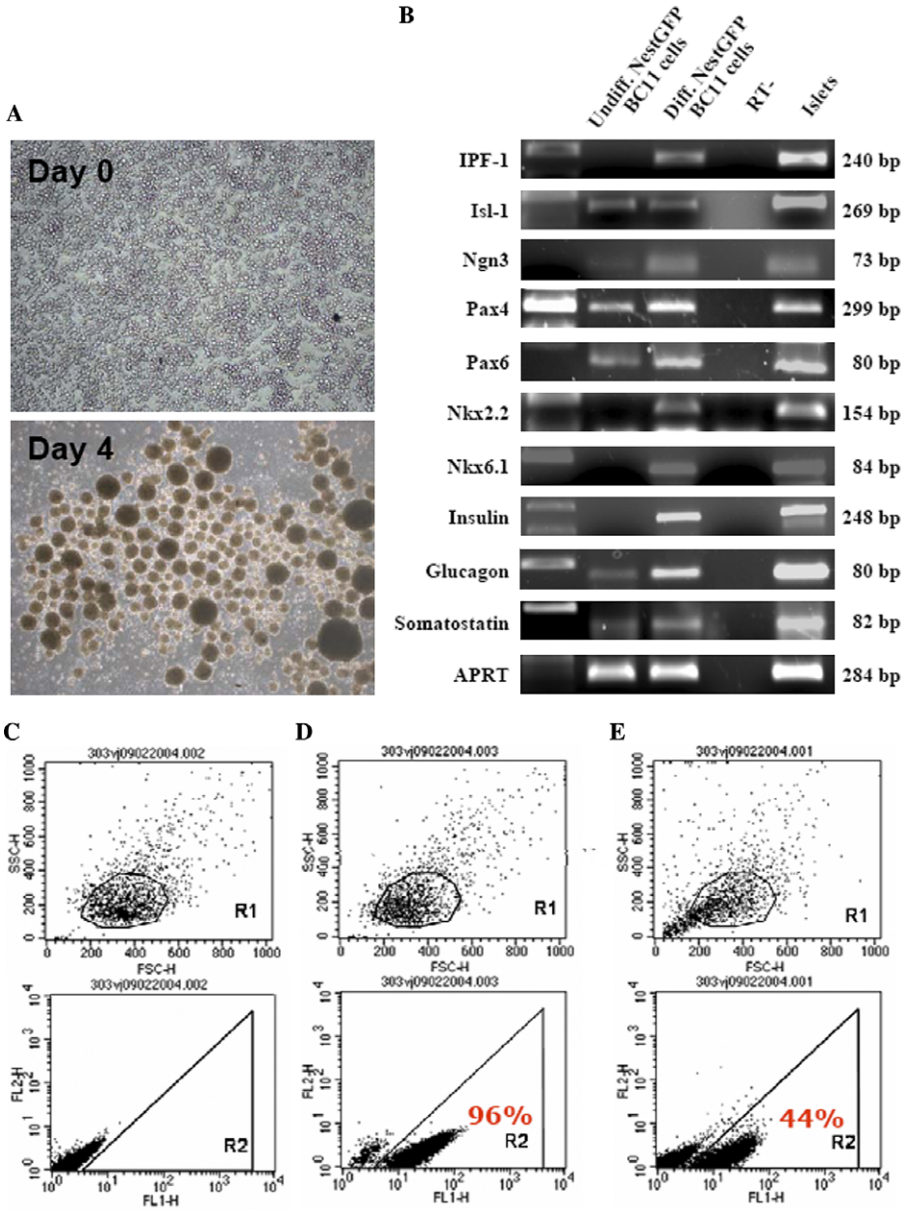

Fig. 4. (A,B) Differentiation of immortalized cells into an endocrine phenotype. (A) After 4 days of culture in differentiation medium, the cells formed islet-like clusters (phase contrast image). (B) Expression of transcription factors known to play an important role in pancreatic endocrine formation including Ipf-1, Isl-1, Ngn3, Pax4, Pax6, Nkx2.2, and Nkx6.1 as well as the mRNA transcripts of the islet hormones insulin, glucagon, and somatostatin (data shown with BC11-BT NestGFP cells). (C–E) Nestin-GFP reporter activity during differentiation. Cells were analyzed by FACS for size (FSC) and aspect (SSC). The FSC vs. SSC 2-D plots were used to set a gate (R1) containing viable cells of similar size for each conditions for further fluorescence analysis. R1-gated cells were then analyzed for GFP fluorescence (FL1, *x*-axis, 4-decade log scale) and autofluorescence (FL2, *y*-axis, 4-decade log scale, lower panel). The percentage and mean of fluorescence intensity were determined in the R2 gate, containing only GFP-expressing cells. BC11-BT cells without NestGFP construct were used as standard for cell size and aspect and as negative control for GFP expression. (C) GFP positive cells before differentiation: 96% (D); GFP positive cells after differentiation: 44% (E).

discrepancy points to one very critical issue in the field, i.e., the differences in culture conditions. As positive control in their studies Humphrey et al. [23] used cultured islet-like cluster from fetal human pancreas. These clusters most likely included already advanced precursors that may require other culture conditions than selected nestin positive cells that failed to differentiate into an endocrine phenotype [23]. Our differentiation medium contains a cocktail of factors known for their beneficial effect on differentiation of precursors into insulin producing cells [19–22]. Moreover, very similar results were obtained when human mesenchymal stem cells from adipose tissue [24] or bone marrow (data not shown, manuscript submitted) were induced to adopt a pancreatic endocrine phenotype.

Differentiation into a hepatic phenotype in vivo

Pancreatic stem or progenitor cells have been shown to adopt a hepatic phenotype in vitro [5] or in vivo [15,25] suggesting the presence of a common hepato-pancreatic

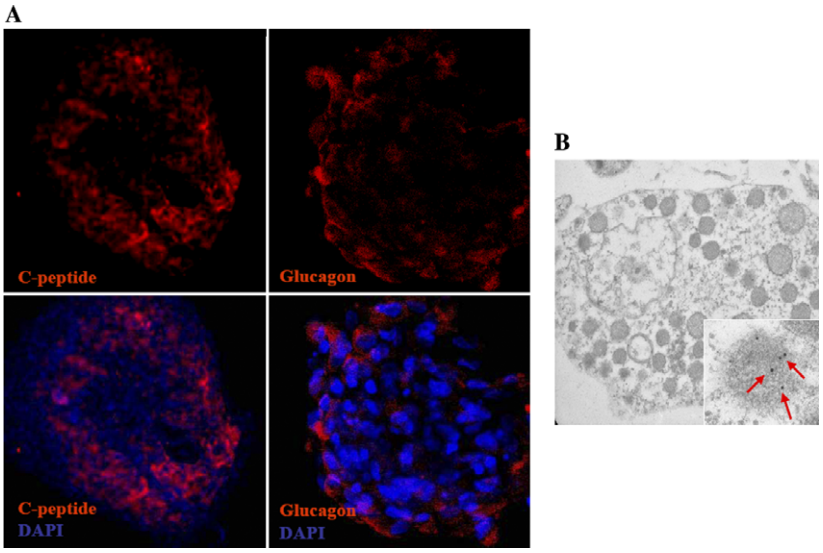

Fig. 5. (A) Immunofluorescence staining of differentiated BC11 cells. Islet-like clusters were stained with anti-C-peptide or glucagon antibody. Nuclei staining was performed with DAPI. Images were obtained with a laser scanning confocal microscope (Zeiss LSM 510). Original magnification 200× for C-peptide image and, 400× for glucagon. (B) Electron microscopy studies revealed formation of granules in differentiated cells in contrast to undifferentiated cells. Red arrows indicate immunogold labeling for C-peptide (inset). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

precursor. In order to test the potential of our islet derived immortalized cells to differentiate into a hepatic phenotype BC11 NestGFP cells were injected directly into the liver of SCID mice. Thirty-five days later human albumin positive cells were found in the livers of three out of 8 transplanted animals (Fig. 6A), indicating that immortalized BC11 NestGFP cells have the potential to acquire a hepatocyte-like phenotype in vivo. RT-PCR studies confirmed the expression of human albumin transcripts in grafted livers (Fig. 6B). Interestingly, it has been reported recently that

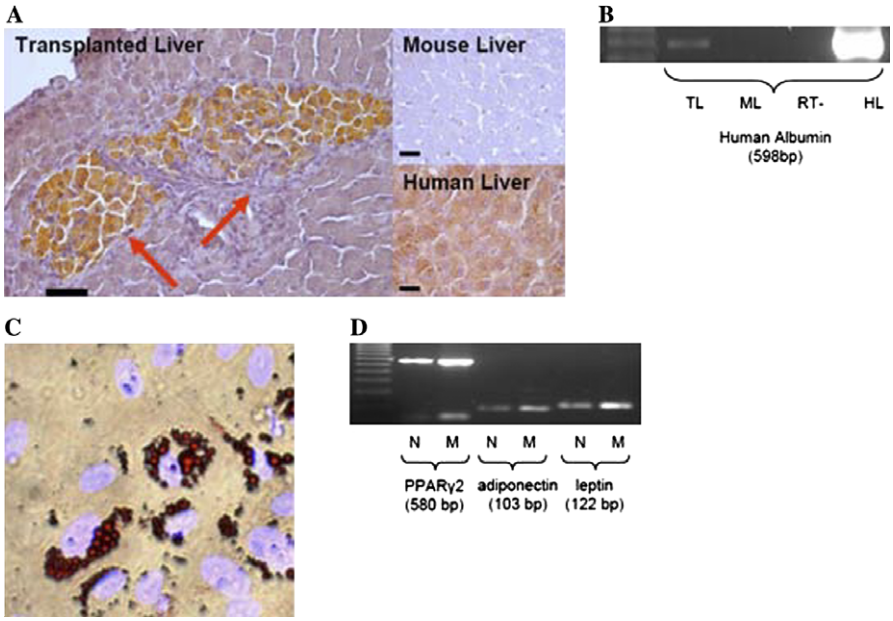

Fig. 6. (A,B) Adoption of a hepatic phenotype in vivo. (A) Thirty-five days after transplantation of immortalized BC11-BT NestGFP cells into SCID mouse liver, immunohistochemistry studies were performed using a monoclonal antibody specific for human albumin. Normal SCID mouse liver was used as negative and human liver as positive control. Original magnification 100×. (B) Detection of human albumin transcript in SCID mouse liver after transplantation of immortalized BC11-BT NestGFP cells (TL). Human liver (HL) was used as positive control. SCID mouse liver (ML) was used as negative control. (C,D) Differentiation into adipocytes. (C) After culturing for 2–3 weeks in adipogenic differentiation medium, immortalized BC11-BT cells formed fat droplets as visualized by Oil Red staining. Nuclear staining was performed with DAPI. Original magnification 400×. (D) Expression of adipocyte-specific genes PPARγ2, adiponectin, and leptin in differentiated cells. The adipocyte-specific genes are not expressed by cultured immortalized cells (not shown). Mesenchymal stem cell derived adipocytes were used as positive control (M).

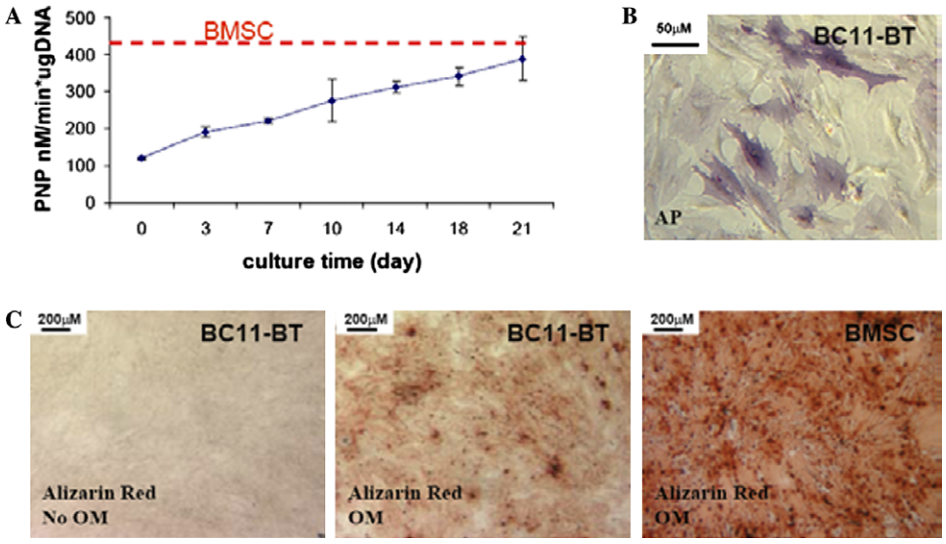

Fig. 7. Differentiation into an osteoblast-like phenotype. (A) Alkaline phosphatase (AP) activity increases during culture of immortalized BC11-BT NestGFP cells in osteogenic medium (OM) reaching levels comparable to those expressed by bone marrow derived mesenchymal stem cells (BMSC). (B) AP positive cells were stained in blue. Original magnification 400×. (C) The BC11-BT NestGFP cells formed foci of mineralization visualized by Alizarin Red after 3 weeks' culture in OM. BMSC cultured in OM were used as positive control, BC11-BT NestGFP cultured without OM were used as negative control. Original magnification 100×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

human bone marrow derived mesenchymal stem cells xenografted directly into rat liver differentiate into human hepatocytes without evidence for fusion [26]. Thus, adoption of a hepatic phenotype in vivo may be a property of mesenchymal stem cells.

Differentiation into adipocytic and osteoblastic phenotypes

Supposing that the immortalized BC11 cells may have a mesenchymal phenotype, we next tried to direct the differentiation into an adipocytic or osteoblastic phenotype using culture conditions normally applied for differentiation of bone marrow derived mesenchymal stem cells or preadipocytes [13,14,16,27]. After 2–3 weeks in adipogenic medium, appearance of fat droplets was observed and paralleled with expression of adipocyte specific genes like PPARγ2, leptin, and adiponectin (Fig. 6C and D). After 3 weeks in osteogenic medium [16], immortalized BC11 cells became positive for alkaline phosphatase and deposited mineralized matrix as shown with the Alizarin staining (Fig. 7A–C).

In our primary islet cell culture the appearance of nestin-expressing cells is most likely the result of FGF2- and EGF-stimulated proliferation of precursor cells that were already present in adult islets of Langerhans. As well, they could derive from bone marrow and belong to a pool of circulating stem cells [28]. Noteworthy, mesenchymal stem/precursor cells from bone marrow [29,30] have been shown to differentiate into insulin-expressing cells in vitro and in vivo [30,31]. Stem cells with a mesenchymal phenotype have been also shown to arise in human islet cultures by de-differentiation of epithelial β-cells induced by growth factors like EGF [32]. The islet derived mesenchymal cells expressed nestin and vimentin and were able to re-differentiate into insulin-expressing cells given the appropriate stimuli [32]. A similar de-differentiation of human islet cells in vitro was also suggested in a recent study although the authors described a de-differentiation into a pancreatic ductal rather than mesenchymal phenotype [33].

The mesenchymal origin of our nestin and Isl-1 positive cells may explain that cell lineage studies did not show pancreatic β-cells originating directly from nestin-expressing cells during development [23,34–36]. But, these studies do not exclude the differentiation of mesenchymal stem cells in insulin producing cells in vitro or in vivo, as shown by the present and previous reports [5,6,20,31,37]. As a limitation of our study the immortalized BC11cells were not yet able to secret insulin in response to high glucose indicating a certain level of developmental immaturity. However, the evidence that these cells can be immortalized with a preserved differentiation potential and without noticeable karyotypic changes, thus probably providing virtually unlimited supplies of insulin producing cells, opens new opportunities for the search of stem cell based therapies of diabetes mellitus.

Acknowledgments

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6.2

In vitro cultured islet-derived progenitor cells of human origin express human albumin in SCID mouse liver in vivo

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In Vitro Cultured Islet-Derived Progenitor Cells of Human Origin Express Human Albumin in Severe Combined Immunodeficiency Mouse Liver In Vivo

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Key Words. Pancreas • Islet of Langerhans • Liver • Stem cell

ABSTRACT

Studies in rodents suggest the presence of a hepatopancreatic stem cell in adult pancreas that may give rise to liver cells in vivo. The aim of the present study was to determine the ability of human islet-derived cells to adopt a hepatic phenotype in vivo. Cultured human islet-derived progenitor cells that did not express albumin in vitro were stained with the red fluorescent dye PKH26 and injected into the liver of severe combined immunodeficiency mice. After 3 or 12 weeks, red fluorescent cells were detected in 11 of 15 livers and were mostly single cells that were well integrated into the liver tissue. Human albumin was found in 8 of 11 animals by immunohistochemistry, and human albumin mRNA was detected in 4 of 10 host livers. The mechanism underlying this phenomenon seems to be transdifferentiation, because human and mouse albumin were found to be expressed in distinct cells in the host liver. *Stem Cells 2004; 22:1134–1141*

INTRODUCTION

During embryogenesis, progenitor cells of pancreas and liver emerge from neighboring areas of the gut endoderm [1]. There is a large body of evidence suggesting that such progenitors with the potential to generate liver cells from pancreatic cells and vice versa may still exist in adult life [2–5]. Using the model of mice with a knockout of the tyrosine catabolic enzyme fumarylacetoacetate hydrolase (FAH), which, without treatment, results in liver cirrhosis, Wang et al. [6] succeeded in correction of liver function by transplantation of cell suspensions from adult pancreas of wild-type animals. With this unique repopulation assay, the authors clearly demonstrated that progenitor cells with the ability not only to replace FAH knockout cells in the liver but also to correct the

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liver function exist in the pancreas, although the exact nature of these cells remains unknown. Interestingly, cells from cultured pancreatic ducts were not able to rescue the failing liver as did the crude pancreatic cell suspension [6], indicating that the presumed hepatopancreatic stem cells reside in areas outside the pancreatic ducts.

Numerous studies have shown the transdifferentiation potential of pancreatic cells of rodents into hepatocytes in vivo. Some very rare cases of human pancreatic cancer with hepatoid phenotype indicated the existence of similar cells in human pancreas [7]. Recently, progenitor cells have been described in rodent and human islets of Langerhans that express the neural stem cell marker nestin [8] and the side-population phenotype marker ABCG2 [9]. The side-population cells in bone marrow represent a particularly potent stem cell population [10]. Interestingly, the human nestin-expressing islet-derived progenitor (NIP) cells were able to adopt a hepatic phenotype in vitro with expression of markers like alpha feto protein and the transcription factor XBP [8]. In contrast to animal data, however, no in vivo studies have been published so far demonstrating transdifferentiation of human pancreatic cells into a hepatic phenotype. Recently, in vivo models for transplantation of human cord blood cells into severe combined immunodeficiency (SCID) mouse liver have been established [11, 12]. Using the model with direct injection of cells into the liver, we demonstrate in the present report that human cells from cultured pancreatic islets of Langerhans engraft into SCID mouse liver and form cells expressing human albumin in vivo.

Materials and Methods

Highly purified human islets are donations from the islet transplantation center in Geneva, Switzerland (Drs. José Oberholzer and Thierry Berney). Growth and expansion of nestin-positive cells were induced by RPMI-1640 medium with 10% fetal calf serum and supplemented with basic fibroblast growth factor and epidermal growth factor (20 ng/ml of each). Nestin-positive cells were characterized by reverse transcription–polymerase chain reaction (RT-PCR) and immunocytochemistry. More than 90% of cells were nestin positive. They were cultured for 4 months in the expansion medium, which was changed every 3–5 days. Trypsinization and reseeding were performed every 10–14 days. All experiments were done with cells of passage 4 through 9. Before transplantation, NIP cells were harvested by trypsinization, washed twice with phosphate-buffered saline (PBS), and stained with the red fluorescent dye PKH26 (Sigma) according to the staining protocol of the supplier (Sigma). The concentration of PKH26 during incubation with NIP cells was 4 μM. Incubation was performed for 4 minutes at 25°C with 10^7 NIP cells per ml. William's E medium with 1% bovine serum albumin (BSA) was used to stop the staining reaction. All centrifugation and washing steps during the staining procedure were performed at room temperature. Afterward, the NIP cells were resuspended in PBS at a concentration of 1.5×10^4 cells, 1.5×10^5, and 7.5×10^5 per 100 μl (Table 1).

The transplantation protocol was approved by the Animal Care and Use Committee in Rheinland-Pfalz, Germany. SCID mice (age, 6–20 weeks; weight, 18–27 g) were obtained from Charles-River (Sulzfeld, Germany). They were fed a standard diet purchased from Sniff (Soest, Germany) and acidified drinking water ad libitum. Before transplantation, mice were anaesthetized by intraperitoneal injection of 61.5 mg/kg ketamine (ketamin-ratiopharm 50, Ratiopharm, Ulm, Germany) and 2.3 mg/kg xylazine (Rompun 2%, Bayer, Leverkusen, Germany). Ketamine and xylazine were combined immediately before administration. The peritoneal cavity was opened directly below the xiphoid cartilage, and the NIP cells were slowly (100 μl in approximately 60 seconds) injected into the parenchyma of the protruding liver lobe using a 26-gauge needle (0.45 × 25, Henke-Sass, Tuttlingen, Germany). Immediately before injection, the cell suspension was warmed to 37°C. Successful injection was approved by a short-term paleness of the liver lobe. Previously, negative findings regarding fluorescence signals, RT-PCR, and immunohistochemistry (see below) have been observed after injection of human mononuclear cells as controls [11].

Three or 12 weeks after transplantation (Table 1), SCID mice were killed by neck dislocation, and after opening the peritoneal cavity, the protruding liver lobe that received the injection was excised. This liver lobe was divided into three parts. One part was fixed in 4% paraformaldehyde for immunohistochemical analysis, and two parts were shock frozen in liquid nitrogen for RNA isolation and fluorescence microscopy. Using a cryotome (CM 3000 cryostat, Leica Instruments GmbH, Nussloch, Germany), 5-μm-thick cryosections were produced. Cryosections were transferred onto Super-Frost Plus slides (Menzel, Braunschweig, Germany), air dried, and immediately analyzed by fluorescence microscopy using a standard filter setup for visualization of PKH26.

For immunohistochemistry, peroxidase was blocked in 7.5% H_2O_2 in methanol for 60 minutes at 4°C. Unspecific binding sites were blocked in 3% BSA for 2.5 hours at 37°C. Afterward, an avidin/biotin-block (Vector Laboratories, Burlingame, CA) was performed as described by the manufacturer. Slides were then incubated with a 1:50 dilution of an affinity purified human albumin antibody produced in goat (Bethyl Laboratories, Montgomery, TX; catalogue No. A80-229A) for 60 minutes at room temperature. Detection of first antibody was performed using commercial Vectastain Elite

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Table 1. Detection of donor-derived cells after transplantation of islet-derived progenitor cells into severe combined immunodeficiency mouse livers

Mouse number	Fluorescence marker	Number of cells transplanted	Time before excision of livers	Red fluorescent cells	Human albumin: immunohistochemistry	Human albumin: reverse transcription–polymerase chain reaction
1	PKH26	15,000	—[a]	—	—	—
2	PKH26	15,000	3 weeks	Negative	—	—
3	PKH26	15,000	3 weeks	Negative	—	—
4	PKH26	15,000	3 weeks	Negative	—	—
5	PKH26	15,000	12 weeks	Negative	—	—
6	PKH26	15,000	12 weeks	Negative	—	—
7	PKH26	15,000	12 weeks	Negative	—	—
8	PKH26	150,000	3 weeks	Positive	—	—
9	PKH26	150,000	3 weeks	Positive	—	—
10	PKH26	150,000	12 weeks	Negative	—	—
11	PKH26	150,000	12 weeks	Positive	—	—
12	PKH26	150,000	3 weeks	Positive	Positive	Positive
13	PKH26	150,000	3 weeks	Negative	Negative	Negative
14	PKH26	150,000	3 weeks	Positive	Negative	Negative
15	PKH26	150,000	3 weeks	Positive	Positive	Positive
16	PKH26	150,000	3 weeks	Negative	Negative	Negative
17	PKH26	150,000	3 weeks	Positive	Positive	Negative
18	PKH26	750,000	3 weeks	Positive	Positive	Positive
19	PKH26	750,000	—[a]	—	—	—
20	—	150,000	3 weeks	—	Positive	Negative
21	—	150,000	3 weeks	—	Positive	Negative
22	—	750,000	3 weeks	—	Positive	Positive
23	—	750,000	3 weeks	—	Positive	—

[a]Mouse died immediately after the operation.

ABC Kit (Vector Laboratories) as described by the manufacturer, followed by a 5-minute incubation with 0.6 mg/mL diaminobenzidine at room temperature. Finally, the sections were counterstained with a 1:5 dilution of Mayer's hemalum (Merck, Darmstadt, Germany).

For fluorescence-immunohistochemistry (confocal microscopy, 630-fold magnification), human albumin was detected using a polyclonal anti-human albumin antibody raised in rabbit (Abcam Ltd.) in a 1:500 dilution. Detection of the primary antibody was performed using a Cy3-labeled secondary antibody against rabbit, raised in donkey (Dianova GmbH) in a 1:1000 dilution. Counterstaining was done using 4',6'-diamidino-2-phenylindole (DAPI) (Molecular Probes) at a concentration of 2.3 μg/1,000 μL. Mouse albumin was detected using a polyclonal fluorescein isothiocyanate–labeled anti-mouse albumin antibody raised in goat (Bethyl Ltd.) in a 1:25 dilution. Counterstaining was done using DAPI (Molecular Probes) at a concentration of 2.3 μg/1,000 μL. A digital overlay was performed of Figures 2B and 2C.

For RT-PCR, homogenization of liver samples was performed by a polytron homogenizer, and total RNA was isolated using RNeasy Midi Kit (Qiagen, Hombrechtikon, Switzerland). Total RNA, 1 μg, was subjected to RT-PCR using Omniscript and Taq PCR core kit (Qiagen). Negative controls without reverse transcriptase enzyme were run in parallel to exclude possible contamination. C-DNA was amplified for 38 cycles (94°C for 30 seconds; annealing temperature 60°C for 60 seconds; 72°C for 60 seconds) using the following human-specific, intron-spanning primers: albumin forward ACTTTTATGCCCCGGAACTC and reverse AGCAGCAGCACGACAGAGTA, ABCG2 forward CACA GGTGGAGGCAAATCTT and reverse TCCAGACACAC-CACGGATAA, SCF forward GGTGGCAAATCTTCCAA

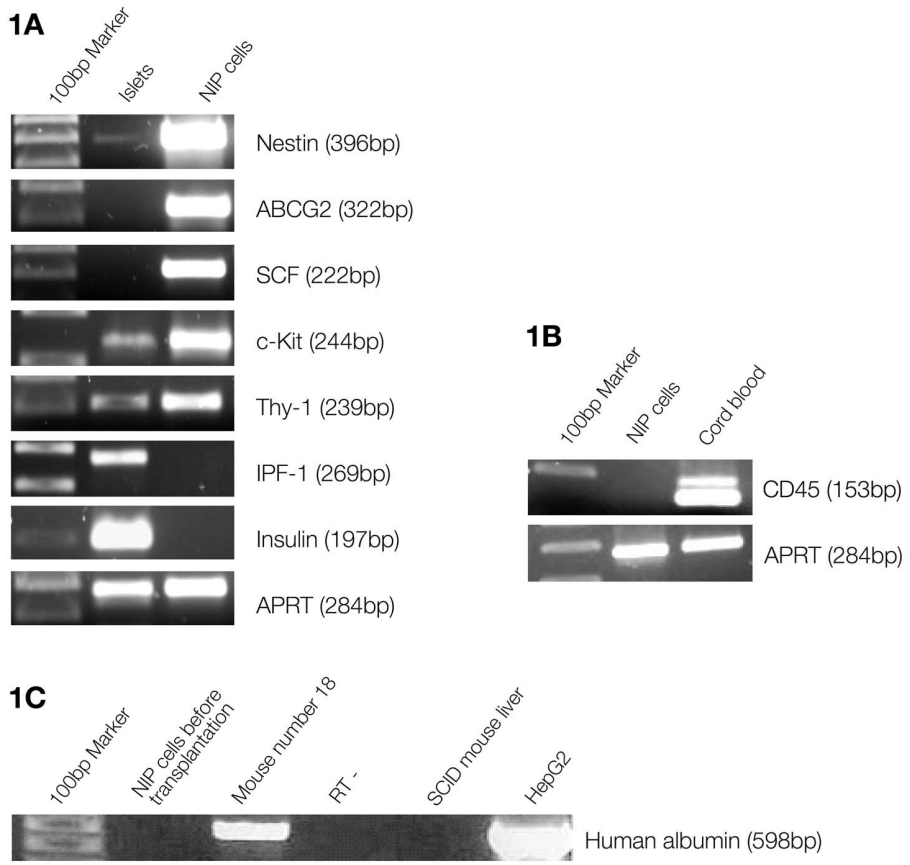

Figure 1. (A): NIP cells not only express the neural stem cell marker nestin but also the side-population marker ABCG2, SCF, c-Kit, and the hepatic stem cell marker Thy-1. NIP cells did not express IPF-1 or insulin. The human housekeeping gene *APRT* was used as positive control for RT-PCR. **(B):** Lack of CD45 expression in NIP cells with positive signal in human cord blood cells. **(C):** Detection of human albumin in SCID mouse liver after transplantation of NIP cells. RT-PCR analysis shows the expression of human albumin mRNA in transplanted SCID mouse number 18. Human albumin is not expressed by cultured NIP cells. HepG2 cells were used as positive control. SCID mouse liver was used as negative control. The origin of all PCR products was confirmed by sequencing. Abbreviations: *APRT*, adenine phosphoribosyltransferase; NIP, nestin-expressing islet-derived progenitor; RT-PCR, reverse transcription–polymerase chain reaction; SCID, severe combined immunodeficiency.

AAG and reverse TCTTTCACGCACTCCACAAG, c-Kit forward GGCATCACGGTGACTTCAAT and reverse GGT TTGGGGAATGCTTCATA, Thy-1 forward GTCCTTTC TCCCCCAATCTC and reverse GGGAGACCTGCAAGAC TGTT, IPF-1 forward CCTTTCCCATGGATGAAGTC and reverse TTGTCCTCCTCCTTTTTCCA, insulin forward CTACCTAGTGTGCGGGGAAC and reverse GCTGGTA GAGGGAGCAGATG, CD45 forward CAGGCAGCAAT GCTATCTCA and reverse CTGTGATGGTGGTGTTG-GAG, and adenine phosphoribosyltransferase (APRT) forward GCGTGGTATTCAGGGACATC and reverse CAGGG CGTCTTTCTGAATCT. Identity of the amplified PCR product was confirmed by sequencing.

Normal human metaphase spreads were prepared from approximately 70% confluent NIP cells in a T25 culture flask by addition of 20 µl colcemid (10 µg/ml, KaryoMax, Gibco-BRL) and incubation for 1.5 hours. Cells were then trypsinized, pelleted, and resuspended in hypotonic solution (KCl-Na-citrate). After fixation with methanol/acetic acid (3:1), typsin/giemsa G-banding was performed according to standard laboratory procedures. Slides were examined with a ZEISS-Axiophot (Zeiss), and images were taken using a CCD camera (Cohu) and specially designed software (Karyotech 2000).

Results

NIP cells expressed beside nestin also the side-population marker ABCG2 as well as SCF, c-Kit, and Thy-1, another potential marker for hepatic stem/progenitor cells (Fig. 1A). These cells were negative for expression of the transcription

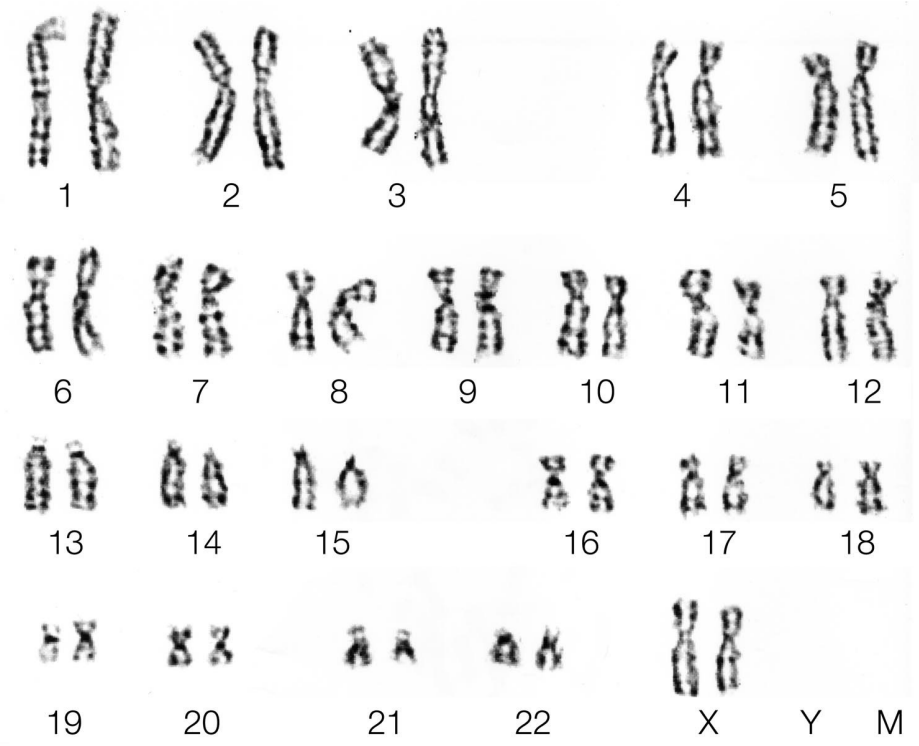

Figure 2. GTG banded metaphase of a nestin-positive cell showing an intact 46,XX karyotype.

factor IPF-1 and insulin but also the specific marker for hematopoietic cells CD45 (Fig. 1B). Before transplantation, no albumin expression was found in cultured NIP cells (Fig. 1C). To exclude numerical or structural chromosomal aberration due to prolonged growth stimulation in vitro, a karyotyping was performed and revealed a normal 46,XX karyotype (Fig. 2).

Transplantation of 1.5×10^4 human NIP cells failed to result in detectable red fluorescent cells that became detectable only after transplantation of 1.5×10^5 cells in three of four animals (Table 1). We next evaluated the impact of time after transplantation on engraftment frequency and found similar results 3 and 12 weeks after transplantation. Cells expressing human albumin were found in liver sections of 8 out of 11 animals. The cells were well integrated into the liver tissue and were predominantly found adjacent to vascular structures (Fig. 3). Transplantation of NIP cells without prior tagging with PKH26 seemed to be more successful and resulted in detection of human albumin-positive cells in all four grafted animals. To analyze fusion as a possible mechanism underlying this phenomenon, immunohistochemistry studies were performed using mouse-specific and human-specific anti-albumin antibodies. In case of fusion, we expected both types of albumin to be expressed in the grafted cell. Using confocal microscopy, expression of human and mouse albumin was found in distinct cells, suggesting that NIP cells did adopt a hepatic phenotype by differentiation induced by surrounding liver tissue rather than fusion (Fig. 4). Additionally, RT-PCR was performed, demonstrating the presence of human albumin mRNA in 4 of 10 animals (Fig. 1C). In the four human albumin–positive livers, the human APRT was also amplified using RT-PCR, although the signal was less abundant than albumin (data not shown). No mononuclear cell infiltration associated with human albumin–positive cells and no neoplasm were observed 3 and 12 weeks after transplantation. In our model, the occurrence of human albumin–positive cells in general, however, was a rare event, with detection of one to three positive cells on every second slice.

Discussion

Adoption of a hepatic phenotype by human NIP cells has been previously described in vitro [8], suggesting that these cells may represent a common hepatopancreatic precursor. In the present study, we show for the first time that cultured NIP cells from human islets of Langerhans express human albumin in vivo when transplanted into SCID mouse liver. During the expansion period, these cells were negative for the albu-

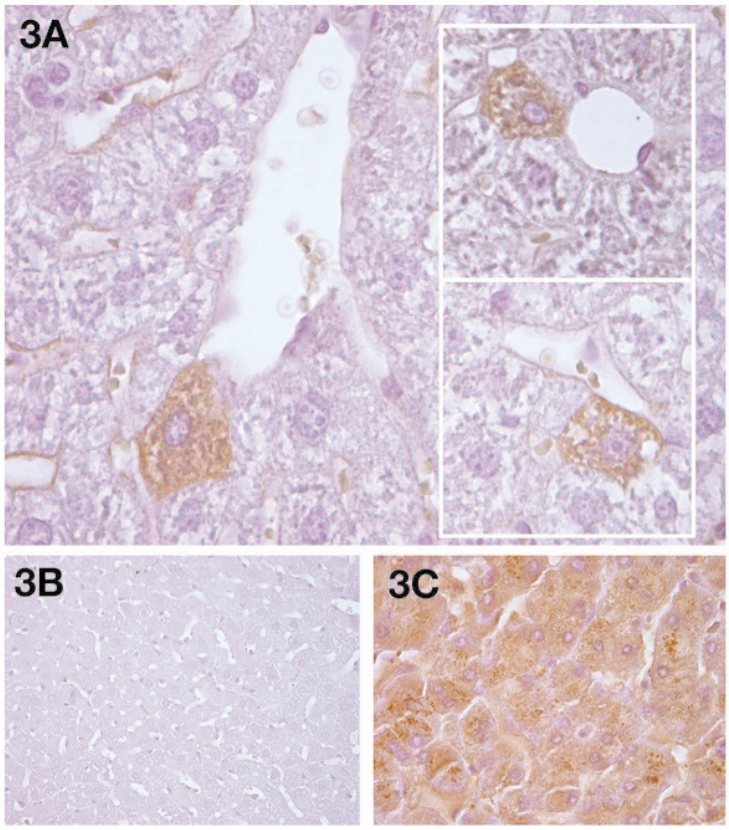

Figure 3. Immunohistochemistry 3 weeks after transplantation of human islet-derived progenitor cells into SCID mouse liver using a monoclonal antibody specific for human albumin and diaminobenzidine (brown) for staining **(A)**, nontransplanted SCID mouse liver as negative control **(B)**, and human liver **(C)** as positive control (bar = 20 μm). Note the proximity of grafted cells to vascular structures.

min transcript and thus acquired this phenotype after injection into the liver only. The immunohistochemistry results clearly show the expression of human albumin after transplantation and demonstrate that these cells are well integrated into the host liver tissue (Fig. 3), although it was in general a rare event, with one to three cells every second slice. The human albumin–positive cells were found mostly as scattered single cells, with rarely also formation of small clusters. Interestingly, we found more human albumin–positive cells after transplantation without labeling with PKH26, probably because of its known cytotoxic effect [13]. The dose-finding experiment showed that at least 1.5×10^5 injected cells are required for detection of human albumin–positive cells, and the best results were obtained with 7.5×10^5 cells, in which human albumin–expressing cells were found in all recipient livers. Interestingly, many of the scattered cells were found adjacent to vascular structures (Fig. 3), as already described by Newsome et al. [12] in their study with infused human cord blood cells. In their report, the appearance of human albumin–positive cells in general was a rare event, with similar efficiency after 4, 6, or 16 weeks [12]. Likewise, we have seen very similar results 3 and 12 weeks after transplantation, although we did not quantify the real transplantation efficiency in this proof-of-principle study. The RT-PCR studies confirmed the immunohistochemistry results in some but not all animals (Fig.1, Table 1). This may be because of the enormous dilution of human mRNA with mouse mRNA in the RT process. The appearance of human albumin–positive cells in the transplanted livers does not necessarily indicate fully functioning hepatocytes, although albumin is the most characteristic protein synthesized by mature liver, accounting for more than 10% of total protein synthesis and the most abundant transcript in hepatocytes [14].

In the elegant studies by Wang et al. [6], a repopulation assay of FAH-deficient animals was used as a gold standard to determine the replacement of liver function by pancreatic stem/progenitor cells in the failing organ. This type of experiment, however, is not yet feasible for studies with human cells using SCID mice as recipients.

The mechanism underlying the adoption of a hepatic phenotype by grafted human NIP cells seems to be transdifferentiation rather than fusion, because expression of mouse

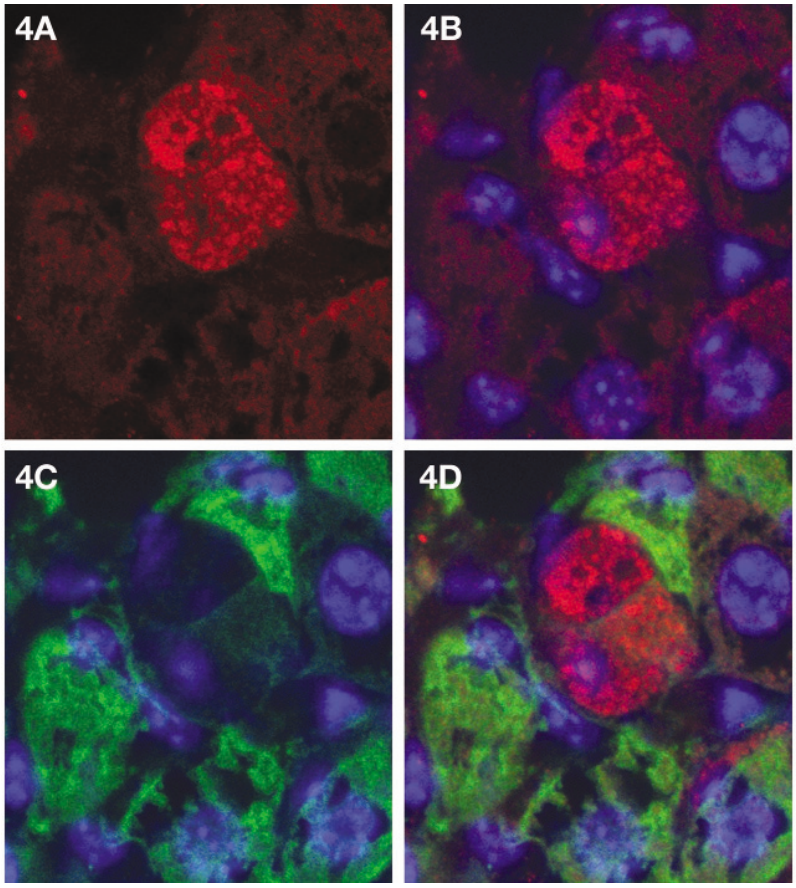

Figure 4. Fluorescence-immunohistochemistry with human and mouse specific antibodies against albumin using confocal microscopy with 630-fold magnification. **(A):** One cell stained with antibodies against human albumin. **(B):** The same cell with additional 4',6'-diamidino-2-phenylindole staining for cell nuclei. **(C):** Albumin staining with antibodies against mouse albumin. **(D):** Digital overlay of human and mouse albumin staining showing no costaining for mouse albumin in the human albumin-positive cell.

and human albumin was found in distinct cells (Fig. 4). In a fused cell, we would expect to find expression of mouse and human albumin in the same cell. Fusion, however, cannot be excluded based on these findings alone, although it seems less likely. In particular, we cannot rule out that fusion was indeed the initial event, followed by reduction division, which restored the cell to its normal (in our case human) diploid state with expression of human albumin only. Reduction division after fusion has been described for hematopoietic cells that fused with hepatocytes [15]. An increasing number of most recent reports demonstrates that cell fusion is a common phenomenon when hematopoietic stem cells engraft into liver tissue [15, 16] and in the central nervous system [17]. Moreover, fusion of human hematopoietic stem cells that were injected in utero in swine were shown to yield transdifferentiation and retroviral transfer among species [18], indicating that fusion of stem cells may be a common phenomenon in vivo. However, transdifferentiation of hematopoietic stem cells into hepatocytes without evidence for fusion was also reported recently [12]. Cell fusion in vivo has been described for hematopoietic stem cells but not pancreatic stem cells. The NIP cells used in our study were negative for the specific marker for hematopoietic cells CD45 (Fig. 1B).

Recently, human nestin-positive islet-derived progenitor cells have been shown to engraft into many tissues, including the liver, of immunocompetent mice [19] without rejection, although the investigators did not analyze the expression of tissue-specific genes of human origin. This remarkable study, however, stresses a particular characteristic of NIP cells as stem/progenitor cells that are not rejected in a xenotransplantation setting [19].

In the present study, expression of human albumin in SCID mouse liver after transplantation of NIP cells is another important in vivo proof for the stem cell potential of these cells.

Summary

Human islet-derived stem cells are capable of adopting a hepatic phenotype in a SCID mouse liver in vivo, suggesting the presence of a hepatopancreatic stem/progenitor cell within or adjacent to the islets of Langerhans. The mechanism underlying this phenomenon seems to be transdifferentiation, although fusion with host hepatocyte cannot be completely ruled out. In the context of these recent findings, one could envision new therapeutic avenues for the treatment of liver cirrhosis using human pancreatic stem/progenitor cells in which such cells could be isolated from pancreatic biopsies and expanded in vitro before trans plantation.

Acknowledgments

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6.3

Human bone marrow-derived mesenchymal stem cells differentiate into insulin, somatostatin and glucagon expressing cells

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In preparation

Human bone marrow-derived mesenchymal stem cells differentiate into insulin, somatostatin and glucagon expressing cells

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Abstract

Replacement of insulin producing cells represents an almost ideal treatment for patients with diabetes mellitus type 1. Here we show that human bone marrow-derived mesenchymal stem cells (MSC) harbour the potential to differentiate into insulin, glucagon and somatostatin expressing cells *in vitro*. MSC from 5 healthy donors and 3 patients with diabetes type 1 were expanded and differentiated using defined culture conditions. Upon differentiation we observed the expression of various pancreatic genes including the transcription factors Isl-1, Ipf-1, Ngn-3, Pax-4, Pax-6, Nkx-2.2 and Nkx-6.1 as well as the islet proteins insulin, glucagon, somatostatin and the glucose transporter glut-2.

Introduction

Stem/progenitor cells with the potential to differentiate into insulin expressing cells were described in pancreatic ducts [1], islets of Langerhans [2,3], the liver [4,5], the central nervous system [6], the spleen [7] and bone marrow [8,9]. Mesenchymal stem cells (MSC) from mouse bone marrow were shown to harbour the potential to differentiate into insulin secreting cells *in vitro* and to reverse hyperglycaemia in an animal model of diabetes [9]. Similarly, mesenchymal precursor cells from mouse spleen were able to regenerate insulin producing cells in a mouse model of autoimmune diabetes [7]. Recently human MSC were described to express at low level the islet transcription factor Nkx-6.1 and to differentiate into insulin producing cells upon adenoviral transduction with vectors over-expressing the transcription factors Ipf-1, Hlxb-9 or Foxa-2 [10]. The mechanisms underlying this apparent developmental plasticity of MSC are unknown. Interestingly, MSC were also shown to bear the potential to adopt a neural phenotype *in vitro* and *in vivo* [11-14] in rodents and humans [13] suggesting a neuro-endocrine developmental capacity of these cells. Expanding MSC express several stem cell marker like stem cell factor (SCF) and Thy-1 [15,16] but also nestin [13,14,17], a gene initially characterized as a marker of neural stem or progenitor cells [18] and later also suggested to be a marker for multipotent pancreatic stem cells [3]. Neural precursor cells express beside nestin also the side population stem cell marker ABCG2 [19,20]. Nestin and possibly ABCG2 expression could therefore represent a possible link between MSC and their ability to differentiate into neuro-endocrine cells. ABCG2 expression was not yet described in human bone marrow derived MSC.

Stem/progenitor cells with the capacity to adopt a pancreatic endocrine phenotype should follow at least in part the complex program of normal pancreas development, for review see [21]. Initiation of pancreas development requires the induction of the transcription factor Ipf-1 that is prerequisite for

pancreas formation in mouse and man [22,23]. Shortly thereafter the transcription factor islet-1 (Isl-1) that is required for generation of endocrine cells is induced at day E9 [24] followed by induction of the transcription factor Ngn-3, another crucial step toward pancreatic endocrine cells [25,26]. In the present study we show that MSC from human bone marrow of non-diabetic subjects as well as patients with longstanding type 1 diabetes are positive for the stem cell markers nestin and ABCG2 and display the potential to activate pancreatic developmental genes in response to defined culture conditions. This includes the transcription factors Ipf-1, Isl-1, Ngn-3, Pax-4, Pax-6, Nkx-2.2, Nkx-6.1 as well as the islet genes insulin, glucagon, somatostatin and glut-2.

Research design and Methods

Isolation and expansion of MSC

Five non-diabetic subjects (age 26 to 64 years) referred to the University Hospital for routine orthopaedic surgery were asked to donate 20 ml bone marrow during the surgical procedure. Three consecutive c-peptide negative patients (age 31, 50 and 53 years) with diabetes mellitus type 1 for 14, 35, and 37 years, respectively donated 20 ml bone marrow that was obtained by aspiration biopsy. All patients gave informed written consent and the study protocol was approved by the local ethics committee for human studies.

After diluting the marrow aspirates with phosphate buffer saline (PBS) at a ratio of 1:4, nucleated cells were isolated using a density gradient solution (Histopaque, Sigma Chemical, Buchs, CH). The cells were cultured in Minimum Essential Medium (MEM) α medium with 5.5 mM glucose containing 10% heat inactivated fetal bovine serum (FBS), 1% HEPES (1M), 1% sodium pyruvate MEM 100 mM, 1% penicillin-streptomycin glutamin (catalogue number 10378016 from Invitrogen AG, Basel, CH). Nucleated cells

were plated at a density of 100,000 cells/cm^2 in the medium supplemented with 5 ng/mL fibroblast growth factor-2 (FGF, R&D Systems, Wiesbaden, Germany) and cultured in a humidified incubator at 37°C, 5% CO_2. FGF was supplemented in order to enrich the fraction of true progenitor cells and to prevent spontaneous differentiation [27]. Medium was changed twice a week. Upon reaching 70-80% confluency, MSC were detached using 0.05% trypsin/ 0.53mM EDTA and replated in culture dishes at a density of 103 cells /cm2 MSC until subconfluency. MSC were then subcultured until the 3rd passage, for a total of 16 to 19 doublings and at an average rate of 0.81 ± 0.07 doublings/day. The described protocol, consistent with most literature in the field, allows to obtain a population of cells negative for the haematopoietic markers CD45, CD14 and CD34 and uniformly positive for the mesenchymal markers CD90, CD44, CD105 and CD166 (data not shown).

MSC culture

After the 3rd passage medium was changed to DMEM with glucose 25 mM (Invitrogen, catalogue number 11971-025) supplemented with 10% heat inactivated FBS, 5 ng/mL FGF, 1% sodium pyruvate MEM 100 mM and 1% penicillin/streptomycin 5000 U/mL (all from Invitrogen AG, Basel, CH). Media and supplements were changed every 72 h. At a confluency of 95%, cells were collected using trypsin (Invitrogen AG, Basel, Switzerland) and washed twice with serum-free DMEM/F12 medium.

For induction of differentiation, MSC were replated at a cell density of 2-3 x 10^5/well and cultured for 3 days in ultra low attachment 6 well plates (Vitaris AG, Baar, Switzerland) allowing the formation of islet-like clusters. MSC from non-diabetic subject 1 were analyzed on two occasions, at passage 4 and passage 7. The gene expression profile of each differentiation study was analyzed every 24 hours for 3 days in every subject or patient. For each differentiation day cells from 4 separate wells were harvested and analyzed

independently and a mean value was calculated. Differentiation medium consists of serum-free DMEM/F12 medium with 17.5 mM glucose in the presence of nicotinamide 10mM, activin-A 2 nM, exendin-4 10nM, hepatocyte growth factor 100 pM and pentagastrin 10 nM (all from Sigma, Basel, Switzerland, except for Pentagastrin that was from Cambridge Laboratories, Newcastle Upon Tyne, UK) as well as B-27 serum-free supplement, N-2 Supplement and 1% penicillin/streptomycin 5,000 U/l (all from Invitrogen AG, Basel, Switzerland). Betacellulin (Sigma Chemical, Buchs, Switzerland) was added in a concentration of 2 nmol/L.

RNA isolation and Reverse transcription (RT)

Total RNA was extracted using TRIzol reagent (Lucerna Chemie AG, Luzern, Switzerland) according to the manufacturer's protocol. RNA samples were treated with DNase (Ambion, Cambridgeshire, UK) in order to remove possible genomic DNA. RNA was quantified spectrophotometrically at 260 nm (Biophotometer, Eppendorf-Vaudaux, Schönenbuch, Switzerland). The quality was assessed by gel electrophoresis on an agarose gel containing ethidium bromide (EtBr, BioRad Laboratories AG, Reinach, Switzerland). 1 μg of total RNA was subjected to reverse transcription (RT) (Omniscript RT kit; Quiagen, Basel, Switzerland). Negative controls without reverse transcriptase enzyme were run in parallel to exclude possible contamination. Human islet RNA was a gift from Wolfgang Moritz, University Hospital Zürich, Switzerland.

Standard polymerase chain reaction (PCR)

PCR was performed on a conventional thermal cycler (TGradient, Biometra, Göttingen, Germany) using PCR Taq core kit (Qiagen) and 2μl of cDNA. Primers were as mentioned in table 1. PCR product identity was confirmed by nucleotide sequencing (Microsynth AG, Balgach, Switzerland).

Quantitative Real-Time polymerase chain reaction (PCR)

Quantitative real-time PCR analysis was performed with the ABI 7000 Sequence (Perkin Elmer, USA) detection system. Specific primers yielding short PCR products suitable for Sybr-Green (Abgene, Epsom, UK) detection were designed with Primer Express software (version 2.0; PE Applied Biosystems, Foster City, CA).The list of primers used is given in table 1. The reaction consisted of 50 μL, containing 25 μL Sybr-Green, 2 μL sense primer (10 μmol/L), 2 μL antisense primer (10 μmol/L), 16 μL H_2O; and 5 μL cDNA. Conditions were set as suggested by the manufacturer. For quantitative analysis of gene expression the standard curve method was used [28]. mRNA expression for each gene of interest was normalized to Hypoxanthine PhosphoRibosyl Transferase (HPRT) expression level that was analyzed with the same cDNA. Results were expressed as the ratio of the respective gene mRNA and HPRT mRNA threshold values. Data are given as percentage expression as compared to day 0.Expression studies were done in quadruplicate (i.e. 4 wells were analyzed independently) for each data point and mean values were calculated for each day. For estimation of mRNA expression as compared to human islets several developmental genes including insulin, glucagon and glut2 were also analyzed in human islets. The relative mRNA expression in differentiated MSC on day 3 was calculated as the ratio of the expression level in differentiated MSC divided by gene expression found in human islets. The product identity was confirmed by sequence analysis. The interindividual variability of gene expression ranged from 9.1×10^{11} to 1.3×10^{14} for HPRT (raw data), from 8.9×10^{-4} to 3.4×10^{-2} for ABCG2 (normalized to HPRT) and from 8.3×10^{-7} to 3.4×10^{-3} for Isl-1 (normalized for HPRT). Similar variation was observed for the other genes.

Peptide measurements

Somatostatin concentration was determined in culture supernatants using a

commercially available somatostatin radioimmunoassay (RIA) kit (functional assay sensitivity: somatostatin 5 pg/tube, Phoenix Pharmaceuticals Inc., Belmont, CA).

Immunocytochemistry

Cultured MSC were transferred into Lab-Tek chamber slides (Nunc, Naperville, IL) and incubated overnight in expansion medium. Islet-like clusters were collected after 3 days in differentiation medium. Some of them were dissociated by trypsin-ethylenediaminetetraacetic acid (EDTA) in order to obtain single cells. Single cells or islet-like clusters were transferred on glass slides coated with poly-L-lysine (Sigma, Buchs, Switzerland) and incubated overnight in DMEM/F12 medium containing 10% FBS allowing them to attach. All cells were then fixed with 4% paraformaldehyde in PBS (pH 7.4) for 30 min at room temperature. After several rinses in PBS, cells were permeabilized with chilled methanol for 10 min. Unspecific binding was prevented by incubation with 10 % heat inactivated FBS in PBS at RT for 30 min. Fixed cultured MSC were incubated with primary antisera for 3 h at RT or overnight at 4°C, rinsed off with PBS and incubated with secondary antisera for 1 h at RT. Fixed islet-like clusters or single cells were incubated with primary antisera for 60-90 min at 37°C, rinsed off with PBS and incubated with secondary goat antisera for 45 min at 37°C. After several washes with PBS, cells were coverslipped with non-fluorescing mounting medium. The primary antibodies used were as follow: Mouse monoclonal antibody 39.4D5 (Developmental Studies Hybridoma Bank, University of Iowa, IA) raised against Isl1 (dilution 1:100), rabbit anti nestin (dilution 1:500) (Chemicon, Juro Supply, Lucerne, Switzerland), rabbit anti-human C-peptide for dispersed cells (dilution 1:500) (Linco, St. Charles, MO), sheep anti-human C-peptide for islet like cluster (dilution 1:100) (Abcam, Cambridge, MA) and rabbit anti- glucagon (dilution 1:100) (Linco, St. Charles, MO). The rabbit anti-

human Ipf1 (dilution 1:1,000) was a generous gift from J.F. Habener, Boston, MA. The secondary antibodies from Molecular Probes (Invitrogen AG, Basel. Switzerland) were goat anti-mouse (dilution 1:1,000), donkey anti-sheep (1:100) and, goat anti-rabbit (dilution 1:1,000). Goat anti mouse IgG and donkey anti-sheep IgG were labeled with alexa fluor 488 dye. Goat anti rabbit IgG was labeled with alexa fluor 546 dye. 4, 6-diamidino-2-phenylindole (DAPI, 5 μg/ml) from Sigma (Basel, Switzerland) was used to label the nuclei (10 min at 37°C). Cells were then examined either by fluorescence microsope (Axiophot, Zeiss, Germany) or by confocal microscope (LSM 510, Zeiss, Germany).The insulinoma cell line INS-1E served as positive control for Isl-1 staining and was kindly provided by Claes Wollheim, Geneva, Switzerland. Hela cells were from ATCC (Manassas, VA).

Results

Isolation of MSC of non-diabetic subjects

Human bone marrow derived MSC from five independent donors were isolated on the basis of adhesion to tissue culture dishes and proliferation in FGF-2 containing culture medium. The initial population of mononuclear cells purified by Ficoll density gradient technique was positive for classical markers of haematopoietic cells like CD45 and c-Kit but also ABCG2 that is known to be expressed in erythroid precursors and natural killer lymphocytes (Fig. 1a). They were negative for Thy-1, nestin and Isl-1. During the process of MSC isolation haematopoietic cells don't adhere to plastic surface and are discarded with medium change leading to loss of CD45 positive cells. Consequently, proliferating MSC were negative for CD45, according to their non-haematopoietic origin and showed a strong expression of SCF and Thy-1, markers typically expressed in MSC (Fig. 1a). C-kit mRNA expression was clearly reduced as compared to initial population of mononuclear cells (Fig.1 a). In addition, MSC expressed nestin, ABCG2 and Isl-1 mRNA (Fig. 1a). Staining for nestin showed that the majority of the cells were positive for this stem cell marker (Fig. 1b). Immunocytochemistry revealed Isl-1 protein in the nuclei of approximately 10% of cultured MSC (Fig. 1d,f).

Induction of differentiation

Induction of differentiation was performed in serum-free medium (17.5 mM glucose) supplemented with exendin-4, pentagastrin, activin-A, betacellulin, nicotinamide and hepatocyte growth factor. Over the three day period we could observe a sharp decrease in the expression level of the stem cell marker ABCG2 (Fig. 2a). This was mirrored by up-regulation of Isl-1, Ipf-1, Ngn-3 and Pax-6 (Fig. 2a). In addition, we observed an up-regulation of the glucose transporter glut-2, insulin, glucagon and somatostatin (Figure 2b).

After having finished the first series of quantitative real time PCR studies we also analyzed the expression of additional transcription factors of the β-cell lineage i.e. Pax-4, Nkx2.2 and Nkx6.1 using standard PCR technique. As compared to undifferentiated cells these developmental markers were also up-regulated at day 3 (Fig. 3).

Analysis of islet proteins in differentiated MSC

Formation of islet like clusters was observed already 24 hours after initiation of the differentiation process (Figure 4a). For immunocytochemistry analysis of Ipf-1 cells in the cluster were dissociated, and stained with the Ipf-1 antiserum. Cells that stained positive for Ipf-1 after 3 days displayed a nuclear staining pattern (Figure 4b). Ipf-1 was not detected in undifferentiated cells (data not shown). C-peptide and glucagon positive cells were found in differentiated islet-like cluster (Fig. 4c-f). Although insulin or glucagon release was not found in differentiated islet like clusters we were able to measure somatostatin release into the culture medium. Somatostatin was detectable already 24 hours after initiation of differentiation and reached plateau levels at days 2 and 3 (Fig. 4g).

When compared to adult human islets the expression levels for the transcription factors Ipf-1, Isl-1, Ngn-3 and Pax-6 found in our islet like cluster reached values of 0.07%, 21%, 290% and 20% respectively, of the expression levels found in human islets. Conversely, the corresponding values for mRNA expression for glut2, somatostatin, glucagon and insulin reached 6.1%, 0.2%, 0.3% and less than 0.01% of the values found in human islets.

Differentiation of bone marrow derived MSC from type 1 diabetic patients

We next sought to determine if MSC from type 1 diabetic patients would also be able to adopt a pancreatic endocrine phenotype. Bone marrow derived MSC from three patients with long-standing type 1 diabetes expressed similar

stem cell markers during the expansion period together with Isl-1 as did MSC from non-diabetic subjects (Fig. 5). In proof of principle experiments induction of differentiation resulted in an up-regulation of the transcripts for Ipf-1, Ngn-3 as well as glut-2, insulin, somatostatin and glucagon as illustrated in table 2. Although all three patients with diabetes expressed Isl-1 and ABCG2 in proliferating MSC and up-regulated insulin, glucagon and somatostatin mRNA during the differentiation period we observed a substantial variation in the expression level of these genes between patients (table 2). C-peptide was also detected with immunocytochemistry on single dispersed cells (Fig. 6)

Discussion

The data presented herein show that human bone marrow-derived MSC are able to adopt a pancreatic endocrine phenotype *ex vivo* in non-diabetic subjects as well as patients with longstanding type 1 diabetes. This was achieved without genetic modification of the cells and in response to defined culture conditions. Initially these cells were cultured in high glucose (25mM) and FGF (5ng/ml) and expressed not only the stem cell markers nestin and ABCG2 but also the transcription factor Isl-1. ABCG2 is a known marker for the side population phenotype stem cells in bone marrow [20] and was recently found in pancreatic islet derived precursor cells and neural stem cells [19,29]. Together with Isl-1 it may thus point to a subpopulation of MSC with a neuro-endocrine developmental potential. During the differentiation experiments a strong down-regulation of ABCG2 gene expression was observed indicating that the cells indeed changed their developmental state from a stem cell to a more differentiated cell type. ABCG2 however is known to be expressed not only in stem cells but also in erythroid precursors and natural killer lymphocytes [20]. Both cell populations are initially co-purified by Ficoll density gradient leading to the positive ABCG2 signal (Fig.1) in the

primary mononuclear cell population that was isolated from the bone marrow and contain mostly haematopoietic cells. During the expansion period however only CD45 negative cells continued to proliferate indicating a non-haematopoietic origin of these cells. In addition classical markers for MSC were up-regulated like Thy-1 and SCF. Similar cells are routinely differentiated into adipocytes or osteoblasts in our institution [30,31] demonstrating their mesenchymal phenotype.

The transcription factor Isl-1 is crucial for the development of pancreatic endocrine cells. Disruption of Isl-1 expression is associated with absence of dorsal mesenchyme and a marked reduction of Ipf-1 gene expression in dorsal epithelium in mice [24]. Embryonic explants of the pancreatic anlage from Isl-1 (-/-) mice did not generate insulin, glucagon or somatostatin positive cells *in vitro* as did explants from Isl-1 (+/-) animals [24]. Isl-1 expression is found at embryonic day 9 in the mouse and is together with Ipf-1 one of the earliest pancreatic markers detected during development [24]. We hypothesize that induction of Isl-1 expression in human MSC may represent the primary critical event that allows adoption of a pancreatic endocrine phenotype. Signals in the culture medium that triggered Isl-1 expression in a subpopulation of MSC could be the high glucose concentration, FBS, FGF-2 or all factors together. The exact mechanisms however, remain to be elucidated.

Beside its crucial role for development of pancreatic endocrine cells Isl-1 is also involved in the development of the central nervous system and the heart [32-35]. Therefore, some of these Isl-1 positive MSC could also represent potential progenitors for motor neurons [32] or cardiomyocytes [35].

Using serum-free medium supplemented with factors known for their beneficial effects on differentiation of pancreatic or hepatic precursors into insulin producing cells [36-39] we have induced the activation of various crucial pancreatic transcription factors including Ipf-1, Ngn-3, Pax-4, Pax-6, Nkx-2.2 and Nkx-6.1 as well as the islet genes insulin, glucagon, somatostatin

and glut-2. The differentiation process *in vitro* described herein may represent -at least in part- replication of ontogeny in response to the defined culture conditions. Noteworthy, these results were obtained in normal subjects as well as in patients with diabetes mellitus type 1 and, without genetic manipulation of MSC. As a limitation, these cells were not yet able to secret insulin or glucagon (data not shown) indicating a certain level of developmental immaturity although they did secrete somatostatin. Somatostatin however is also produced by neural cells and formation of such neural cells cannot be excluded in our cell culture system. Viewing the very low expression of insulin as compared to human islets we are still far away from clinically meaningful insulin production in bone marrow derived MSC. But, for the first time we show that cultured human MSC may have such potential without genetic modification.

The real time PCR studies over 3 days revealed that many pancreatic genes were activated already 24h after induction of differentiation. These profound changes in the transcriptional program occurred surprisingly fast. But, observations of early activation of insulin gene expression were also made in mice *in vivo* in response to hyperglycaemia [40]. Here the authors found insulin positive cells in the bone marrow and adipose tissue of hyperglycaemic mice already after 3 days. These cells could represent MSC as described in the present study and our previous report showing induction of pancreatic developmental genes in human adipose tissue derived MSC [28] and they could share properties of MSC isolated from pancreatic islets [41].

Several studies addressed the possibility that bone marrow derived stem cells could contribute to β-cell turnover *in vivo*. Some of them presented positive results and suggested the presence of a circulating pool of stem cells that could participate in the process of β-cell neogenesis [8]. These results however

were not confirmed by others [42] and a recent report questioned the entire concept of β-cell stem/progenitor cells with studies using genetic lineage tracing experiments [43]. With this approach it has been shown that pre-existing β-cells rather than adult stem/progenitor cells retained a proliferative capacity and may thus represent the major source of new β-cells in adult life, at least in mice [43]. While *in vivo* studies are not conclusive regarding the role of adult stem cells for generation of new β-cells this does not exclude the differentiation of adult stem cells into insulin producing cells *in vitro*. It has been shown that mouse mesenchymal stem cells from bone marrow cultured in high glucose over 4 months induced several β-cell specific genes including insulin and glut-2 [9]. These cells were also able to reverse hyperglycaemia in an animal model of diabetes although other crucial transcription factors like Pax4 and Isl-1 were not expressed [9]. In accordance with this report by Tang et al. our human MSC are also CD45 negative and express nestin during the expansion period.

In summary Isl-1 positive MSC can be isolated from human bone marrow and are able to adopt a pancreatic endocrine phenotype in non-diabetic as well as type 1 diabetic subjects. These cells could be used as a human model to study development of pancreatic endocrine cells *ex vivo* and may help to develop stem cell based therapies for diabetes mellitus type 1.

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Figure legends

Figure 1.

Expression of stem cell markers in mononuclear cells and MSC. *(a) Presence of CD45, c-kit, ABCG2, SCF, Thy1, nestin and Isl-1 mRNA was analyzed by RT-PCR in freshly isolated mononuclear cells (Ficoll separation) and after the 6^{th} passage in expansion medium. (b) Immunocytochemistry for nestin, (c) HeLa cells served as negative control.for nestin antibody. (c-g) Immunocytochemistry for the transcription factor Isl-1 (panels d and f, magnification x 200). Arrows indicate positive staining in nuclei of MSC. Nuclei were counterstained with DAPI. Positive control was carried out with INS1E cells (right panels e and g).*

Figure 2.

Induction of pancreatic developmental genes in MSC in response to defined culture conditions in non-diabetic subjects. *Gene expression was monitored every 24h for 3 days. Data are normalized to HPRT and expressed as percentage expression as compared to day 0. Results are means ± SEM of quadruplicates of six independent experiments from five independent donors. (a) mRNA expression of the side population marker ABCG2 and the pancreatic transcription factors, Isl-1, Ipf-1, Ngn-3 and Pax-6 was analyzed by real-time PCR. (b) Induction of glut-2, insulin, somatostatin and glucagon (logarithmic scale).*

Figure 3.

Expression of Pax4, Nkx2.2 and Nkx6.1 mRNA. *Analysis was performed by conventional PCR before (day 0) and 3 days after induction of differentiation. This experiment was repeated 3 times with MSC from 3 different donors, who were already analyzed with realtime PCR.*

Figure 4.

Analysis of islet like clusters generated from MSC. *(a) Phase contrast image of islet like clusters after differentiation of MSC (magnification x 200). For immunocytochemistry islet-like clusters were collected after 3 days in differentiation medium. Some of them were dissociated by trypsin-EDTA to obtain single cells that were stained for Ipf-1 (b). Undifferentiated MSC were negative for Ipf-1. (c-d) Staining of differentiated clusters for c-peptide and glucagon (magnification x 400). The panels (e-f) show the non-specific affinity of the secondary antibodies. Images were obtained with a laser scanning confocal microscope. Nuclear staining in blue with DAPI. (g) Somatostatin release during the differentiation period was measured with RIA in supernatants collected each day (n=8).*

Figure 5.

Expression of stem cell markers in MSC from Type 1 diabetic patients. *(a) Presence of CD45, c-kit, ABCG2, SCF, Thy1, nestin and Isl-1 mRNA was analyzed by RT-PCR after the 6^{th} passage in expansion medium.*

Figure 6.

Immunocytochemistry for c-peptide in differentiated MSC of patients with type 1 diabetes (DBM1, DBM2 and DBM4). *The c-peptide positive cells were scattered single cells. Staining for c-peptide was absent in all MSC before induction of differentiation.*

Table 1.

Primer sequences for RT-PCR. All primers were run at 60ºC.

gene	sense primer	antisense primer	amplicon	accession number	cycles
HPRT	5´-TCAGGCAGTATAATCCAAAGATGGT-3´	5´-AGTCTGGCTTATATCCAACACTTCG-3´	85bp	M26434	40
Insulin	5´-GCAGCCTTTGTGAACCAACA-3´	5´-TTCCCCGCACACTAGGTAGAGA-3´	69bp	NM_000207	40
Ipf-1	5´-TGATACTGGATTGGCGTTGTTT-3´	5´-TCCCAAGGTGGAGTGCTGTAG-3´	70bp	NM_000209	40
ABCG2	5´-GGTTACGTGGTACAAGATGATGTTG-3´	5´-AGCCGAAGAGCTGCTGAGAA-3´	80bp	AY289766	40
Pax-4	5´- TCTCCTCCATCAACCGAGTC -3´	5´- GTTGGAAAACCAGACCCTCA -3´	299bp	AF043978	40
Pax-6	5´-TGCGACATTTCCCGAATTCT-3´	5´-GATGGAGCCAGTCTCGTAATACCT-3´	81bp	NM_001604	40
Nkx2.2	5´- TCTACGACAGCAGCGACAAC -3´	5´- TTGTCATTGTCCGGTGACTC -3´	154bp	O95096	40
Nkx6.1	5´- TCTTCTGGCCCGGAGTGA-3´	5´- CCAACAAAATGGATCCTTGATGA-3´	84bp	NM_006168	40
Isl-1	5´-CAACTGGTCAATTTTTCAGAAGGA-3´	5´-TTGAGAGGACATTGATGCTACTTCAC-3´	75bp	NM_002202	40
Nestin	5´- CGTTGGAACAGAGGTTGGAG -3´	5´- TAAGAAAGGCTGGCACAGGT -3´	396bp	BC032580	40
Glut-2	5´-AGCACTTGGCACTTTTCATCAG-3´	5´-GCCCAAGATAAATTCAAGACCAAT-3´	82bp	J03810	40
Ngn-3	5`-CTATTCTTTTGCGCCGGTAGA-3`	5`-CTCACGGGTCACTTGGACAGT-3`	73bp	NM_020999	40
Somatost	5´-GATGCCCTGGAACCTGAAGA-3´	5´-CCGGGTTTGAGTTAGCAGATCT-3´	82bp	BC032625	40
Glucagon	5´-CCCAAGATTTTGTGCAGTGGTT-3´	5´-CAGCATGTCTCTCAAATTCATCGT-3´	80bp	NM_002054	40
Thy-1	5´-GTCCTTTCTCCCCCAATCTC-3´	5´-GGGAGACCTGCAAGACTGTT-3´	239bp	NM_033209	40
SCF	5´-GGTGGCAAATCTTCCAAAAG-3´	5´-TCTTTCACGCACTCCACAAG-3´	222bp	BC074725	40
c-kit	5´-GGCATCACGGTGACTTCAAT-3´	5´-GGTTTGGGGAATGCTTCATA-3´	244bp	L04143	40
CD45	5´-CAGGCAGCAATGCTATCTCA-3´	5´-CTGTGATGGTGGTGTTGGAG-3´	153bp	Y00638	40

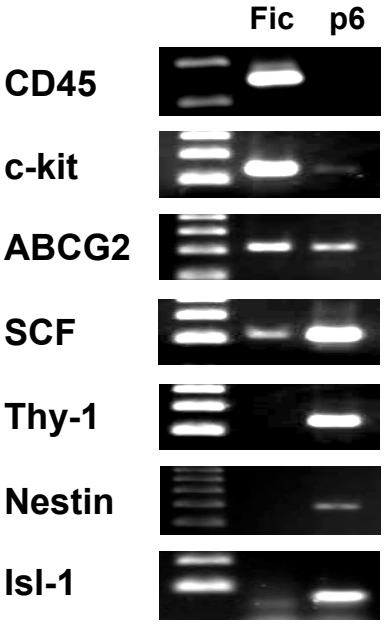
Table 2.

Quantitative real time PCR analysis of pancreatic developmental genes in MSC of three type 1 diabetic patients during the 3 days differentiation period. *Values are given as percentage expression as compared to day 0. Each experiment was run in quadruplicate. In patient 3 Ipf-1 and Glut-2 expression were not detectable (ND) on day 0. Therefore day 1 was taken as reference for comparison with days 2 and 3.*

	patient 1	patient 2	patient 3
ABCG2 day0	100	100	100
ABCG2 day1	41	21	116
ABCG2 day2	14	53	4
ABCG2 day3	49	1	24
IPF-1 day0	100	100	ND
IPF-1 day1	1328	168	100
IPF-1 day2	1990	0	242
IPF-1 day3	2746	9431	16
ISL-1 day0	100	100	100
ISL-1 day1	53	71	109
ISL-1 day2	38	210	117
ISL-1 day3	67	135	228
NGN-3 day0	100	100	100
NGN-3 day1	221	112	1022
NGN-3 day2	417	4455	1521
NGN-3 day3	3463	7708	1501
Pax-6 day0	100	100	100
Pax-6 day1	110	81	4889
Pax-6 day2	112	81	894
Pax-6 day3	61	5	1678
Glut-2 day0	100	100	ND
Glut-2 day1	233	201	100
Glut-2 day2	251	1933	185
Glut-2 day3	2068	2599	313
SRIF day0	100	100	100
SRIF day1	58	220	1402
SRIF day2	121	248	25636
SRIF day3	444	83	9064
glucagon day0	100	100	100
glucagon day1	265	251	1366
glucagon day2	378	8155	16980
glucagon day3	1049	974	4658
Insulin day0	100	100	100
Insulin day1	99	850	56
Insulin day2	263	198	5743
Insulin day3	865	166805	7377

Figure 1

a



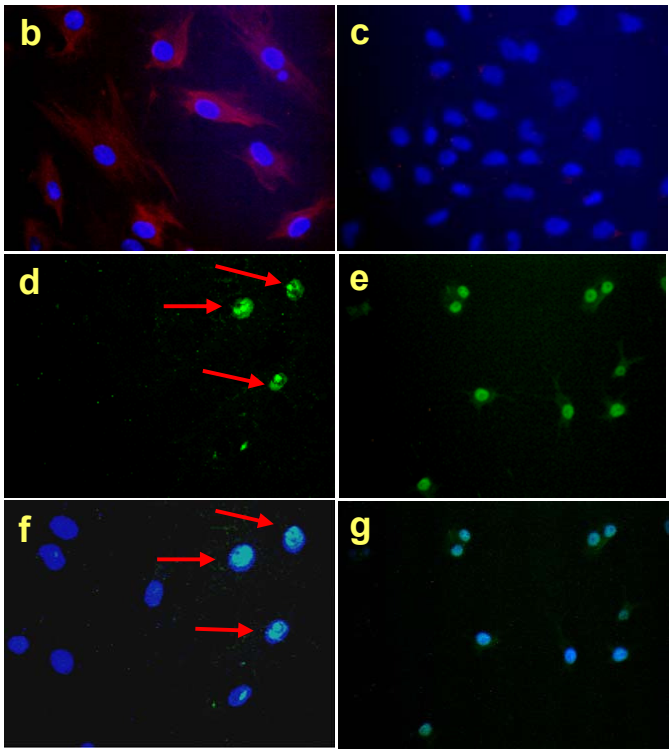


Figure 2

a

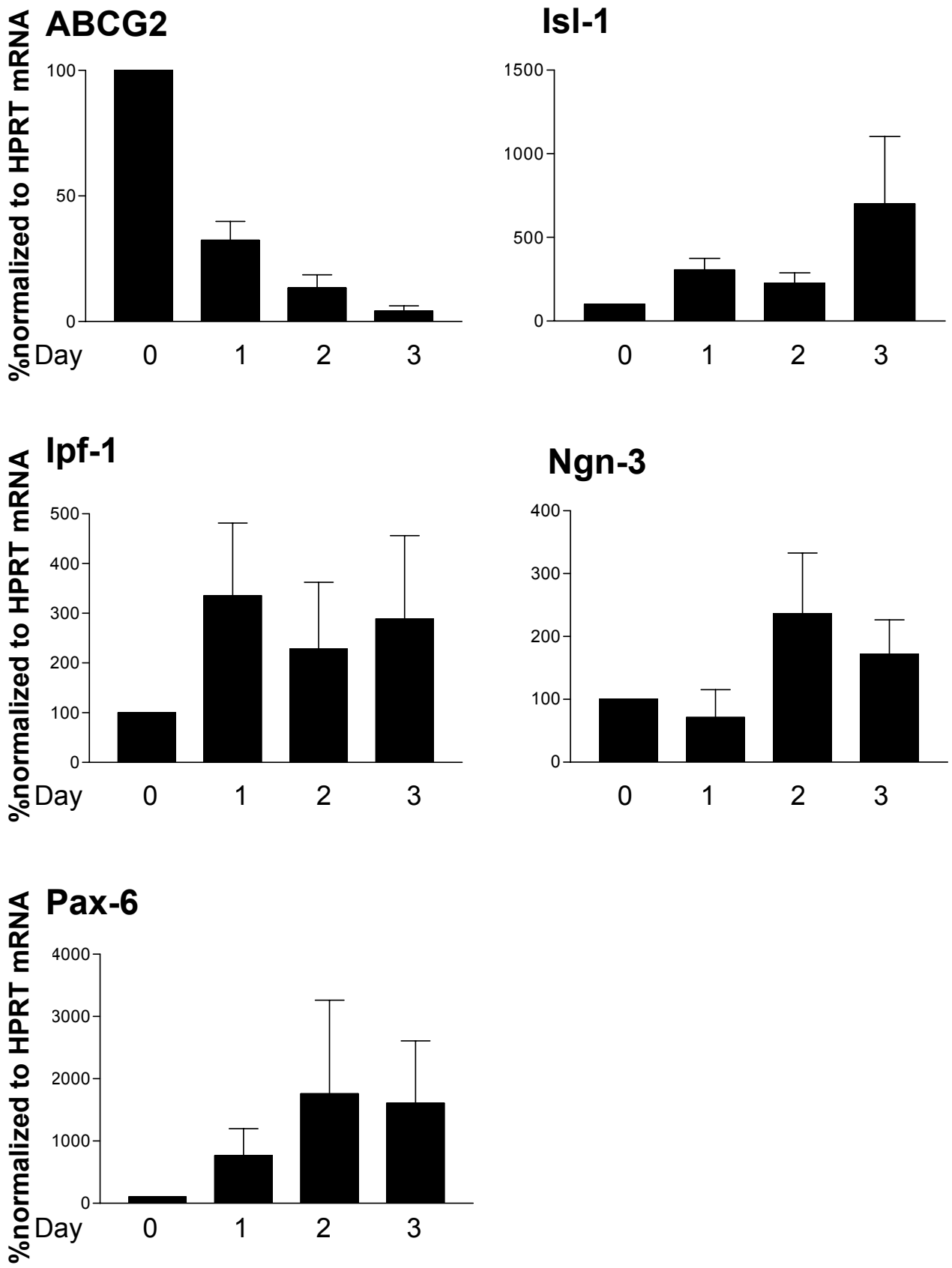

ABCG2
%normalized to HPRT mRNA
100
50
0
Day 0 1 2 3
Isl-1
1500
1000
500
0
0 1 2 3
Ipf-1
%normalized to HPRT mRNA
500
400
300
200
100
0
Day 0 1 2 3
Ngn-3
400
300
200
100
0
0 1 2 3
Pax-6
%normalized to HPRT mRNA
4000
3000
2000
1000
0
Day 0 1 2 3

Figure 2

b

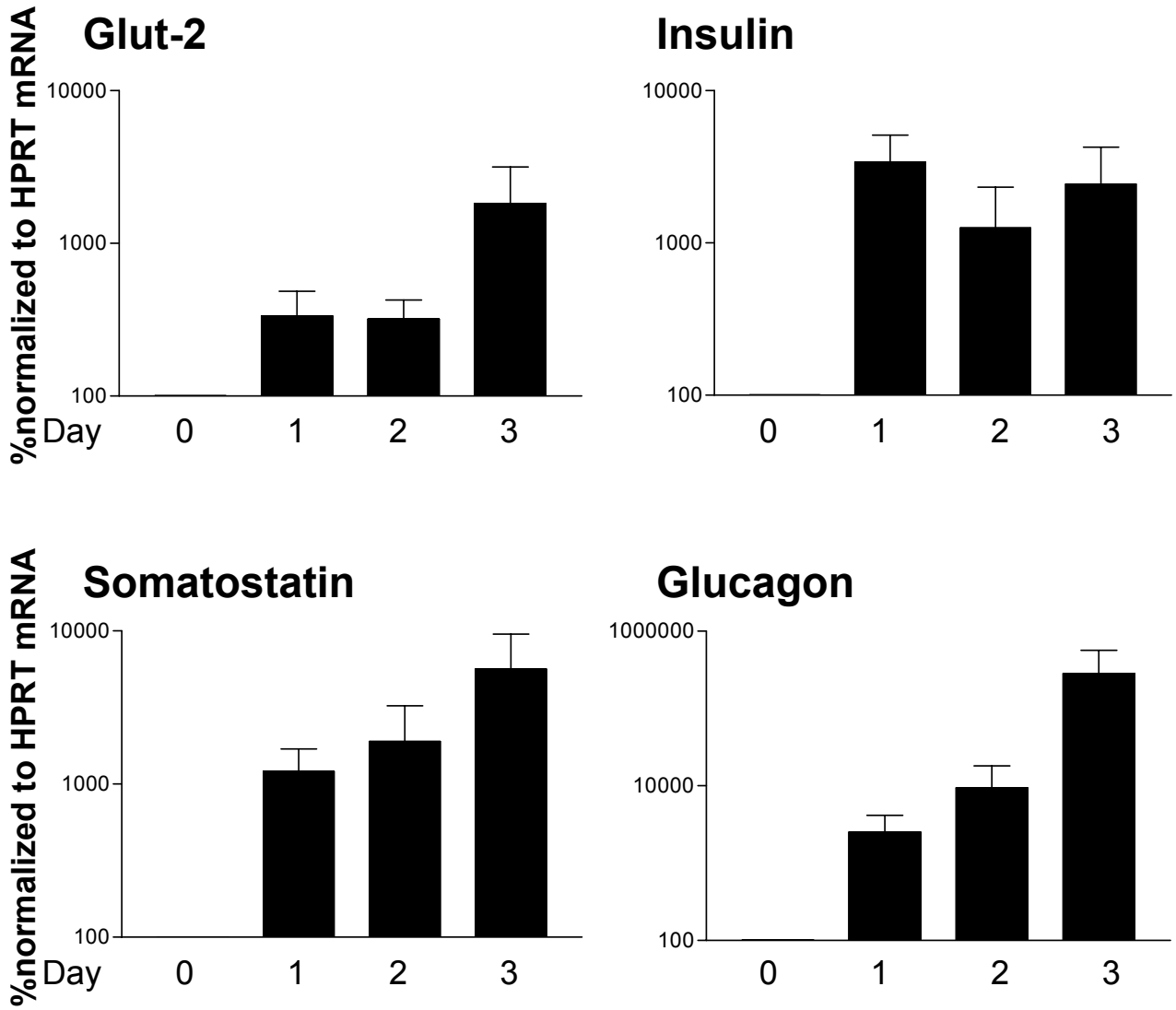

Glut-2
Insulin
Somatostatin
Glucagon
%normalized to HPRT mRNA
10000
1000
100
1000000
Day
0
1
2
3

Figure 3

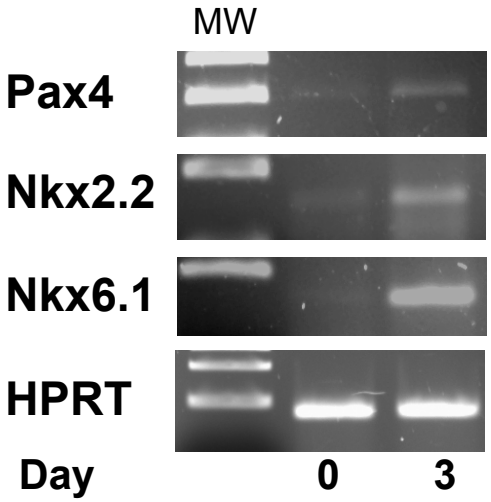


Figure 4

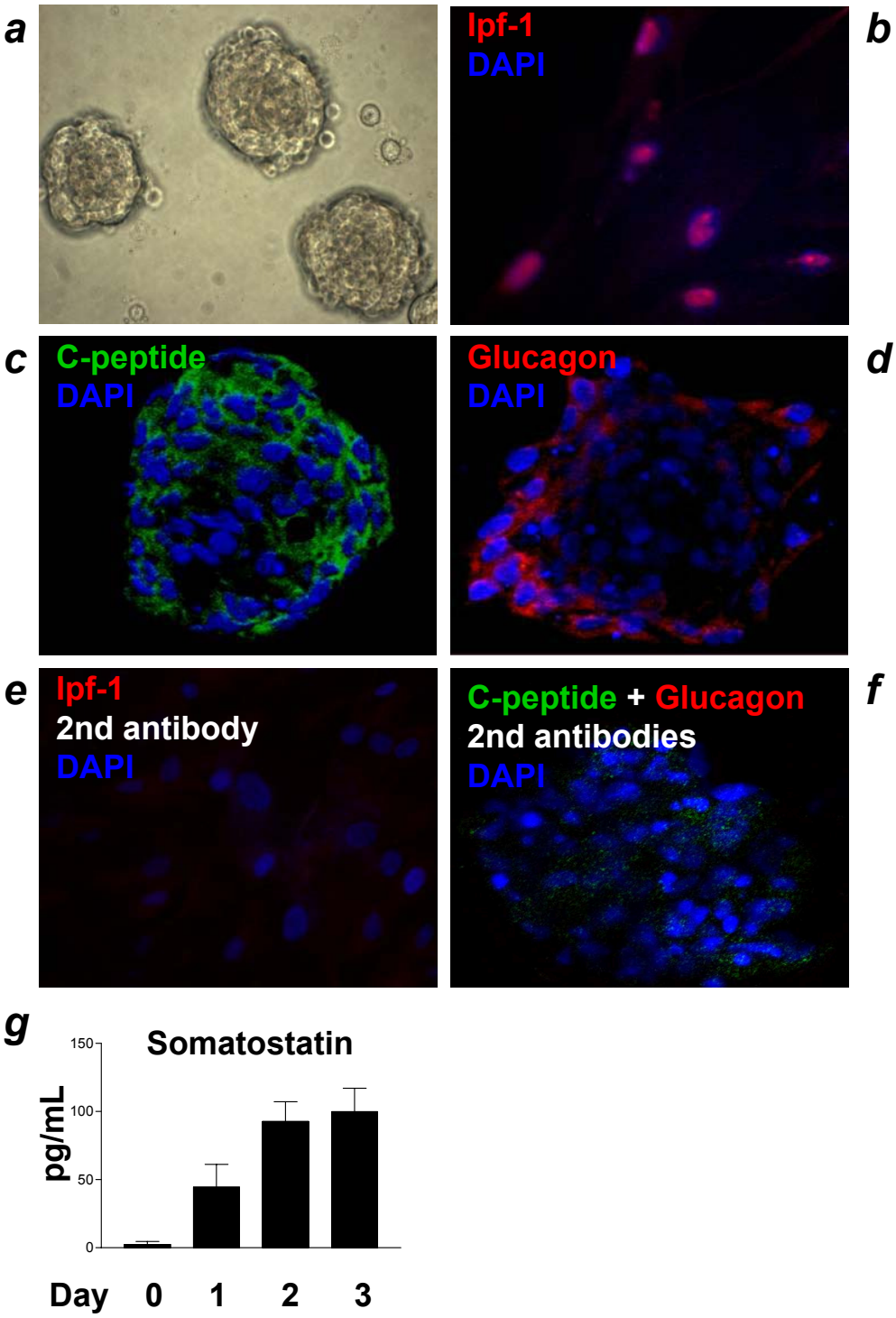

a
b
Ipf-1
DAPI
c
C-peptide
DAPI
d
Glucagon
DAPI
e
Ipf-1
2nd antibody
DAPI
f
C-peptide + Glucagon
2nd antibodies
DAPI
g
Somatostatin
pg/mL
150
100
50
0
Day 0 1 2 3

Figure 5

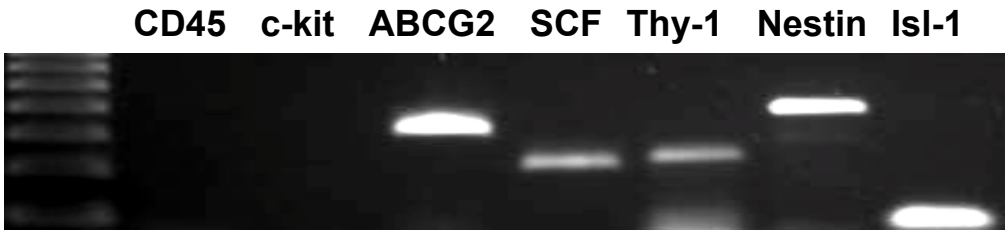
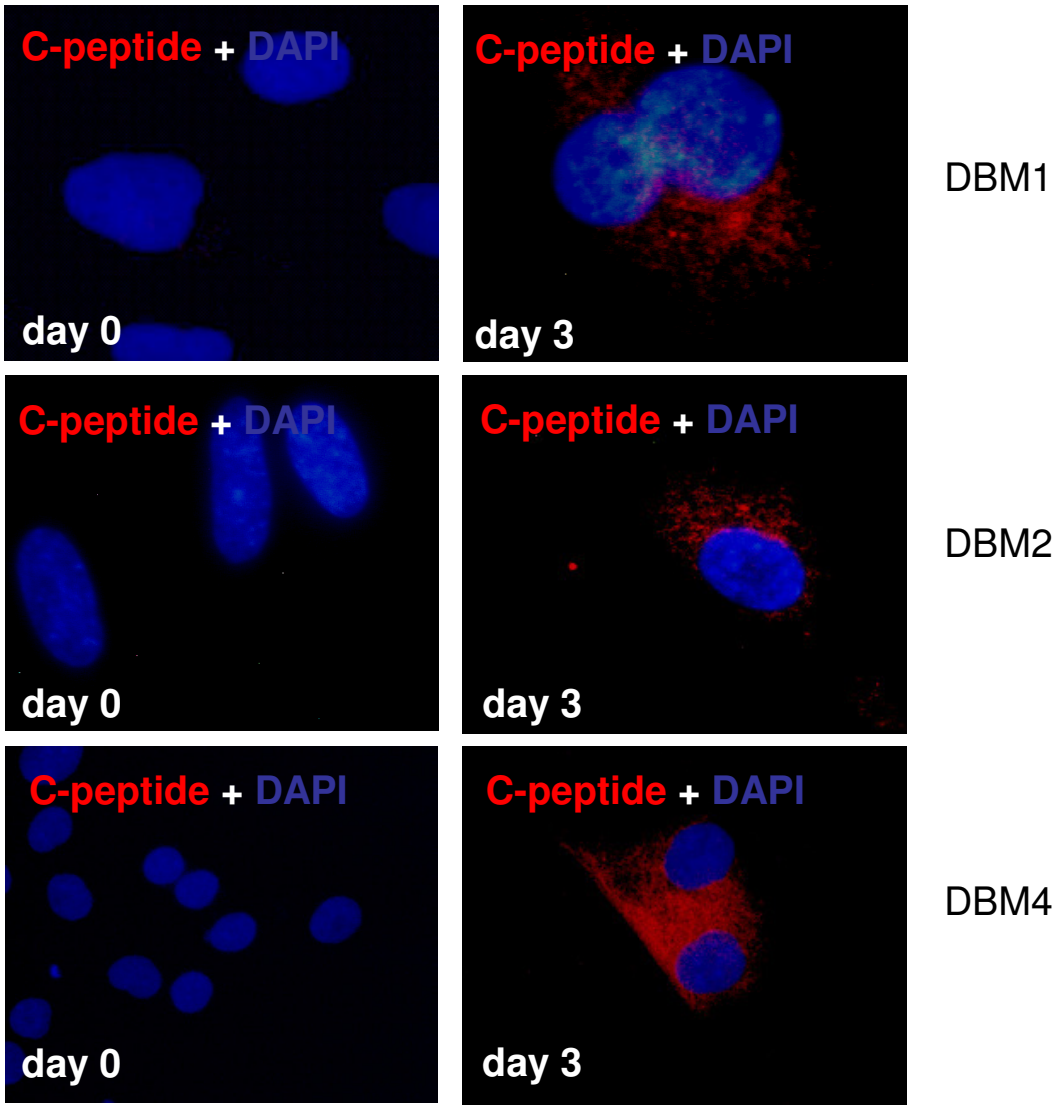


Figure 6


C-peptide + DAPI
day 0
C-peptide + DAPI
day 3
DBM1
C-peptide + DAPI
day 0
C-peptide + DAPI
day 3
DBM2
C-peptide + DAPI
day 0
C-peptide + DAPI
day 3
DBM4

6.4

Human adipose tissue-derived mesenchymal stem cells differentiate into insulin, somatostatin and glucagon expressing cells

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Human adipose tissue-derived mesenchymal stem cells differentiate into insulin, somatostatin, and glucagon expressing cells

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Abstract

Mesenchymal stem cells (MSC) from mouse bone marrow were shown to adopt a pancreatic endocrine phenotype in vitro and to reverse diabetes in an animal model. MSC from human bone marrow and adipose tissue represent very similar cell populations with comparable phenotypes. Adipose tissue is abundant and easily accessible and could thus also harbor cells with the potential to differentiate in insulin producing cells. We isolated human adipose tissue-derived MSC from four healthy donors. During the proliferation period, the cells expressed the stem cell markers nestin, ABCG2, SCF, Thy-1 as well as the pancreatic endocrine transcription factor Isl-1. The cells were induced to differentiate into a pancreatic endocrine phenotype by defined culture conditions within 3 days. Using quantitative PCR a down-regulation of ABCG2 and up-regulation of pancreatic developmental transcription factors Isl-1, Ipf-1, and Ngn3 were observed together with induction of the islet hormones insulin, glucagon, and somatostatin.

Keywords: Mesenchymal stem cells; Isl-1; Human; Adipose tissue; Nestin; ABCG2; Differentiation; Insulin; Glucagon

Mesenchymal stem cells have been initially described as clonal, plastic adherent cells from bone marrow [1] capable of differentiating into adipocytes, chondrocytes, and osteoblasts [2,3]. They have been later identified in various other tissues including muscle, brain, and adipose tissue [4–6]. In addition to their ability to differentiate into adipocytes, osteoblast, and chondrocytes, these stem cells were also found to adopt a neural and hepatic phenotype in vitro and in vivo [7–12]. Proliferating MSC express the stem cell marker nestin [9,11,13], a gene initially characterized as a marker of neural stem or progenitor cells [14] and later also suggested to be a marker for multipotent pancreatic stem cells [15]. MSC from mouse bone marrow were recently shown to harbor the potential to differentiate into insulin secreting cells in vitro and to reverse hyperglycemia in an animal model of diabetes [16]. Similarly, mesenchymal CD45-negative precursor cells from mouse spleen were able to regenerate insulin producing cells in a mouse model of autoimmune diabetes [17]. The mechanisms underlying this apparent developmental plasticity of MSC are unknown. MSC from human bone marrow and adipose tissue represent a very similar cell population with comparable phenotypes [6,18–20]. Thus, MSC with the potential to adopt a pancreatic endocrine phenotype could also exist in human adipose tissue. In the light of the actual worldwide diabetes epidemic, the generation of insulin producing cells from adipose tissue-derived stem cells represents an attractive treatment option for patients who have lost their residual insulin production. In the present study, we show that MSC from human adipose tissue express the stem cell markers nestin and ABCG2, and display the potential to activate pancreatic developmental genes in response to

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defined culture conditions. This includes the transcription factors Ipf-1, Isl-1, Ngn-3, and Pax-6 as well as the islet proteins insulin, glucagon, and somatostatin.

Materials and methods

Isolation and expansion of human adipose tissue-derived MSC. Human adipose tissue was obtained from patients undergoing plastic surgery in accordance with the Local Ethics Committee (University Hospital Basel, Switzerland). Adipose tissue-derived MSC were isolated and expanded as previously described [21]. Culture medium contained DMEM supplemented with 10% fetal bovine serum (FBS) and 5 ng/mL fibroblast growth factor (bFGF), 1% sodium pyruvate MEM 100 mM, and 1% penicillin/streptomycin 5000 U/mL (all from Invitrogen, Basel, Switzerland). MSC were cultured in 175 cm^2 Flasks (Becton–Dickinson AG, Basel, Switzerland). Medium was changed twice a week. At a confluence of 95%, cells were collected using trypsin (Invitrogen AG, Basel, Switzerland) and washed twice with DMEM/F12 medium.

For induction of differentiation, the cells were seeded at a cell density of $2–3 \times 10^5$/well of a 6-well plate and cultured for 3 days in ultra-low attachment plates (Vitaris AG, Baar, Switzerland). MSC from four donors were analyzed at passages 4–7. The gene expression profile of each differentiation study was analyzed every 24 h for 3 days in every subject. For each differentiation day cells from four separate wells were harvested and analyzed independently and a mean value was calculated. Differentiation medium consisted of serum-free DMEM/F12 medium with 17.5 mM glucose in the presence of nicotinamide 10 mM, activin-A 2 nM, exendin-4 10 nM, hepatocyte growth factor 100 pM, and pentagastrin 10 nM (all from Sigma, Basel, Switzerland) as well as B-27 serum-free supplement, N-2 Supplement, and 1% penicillin/streptomycin 5000 U/L (all from Invitrogen AG, Basel, Switzerland).

RNA isolation and reverse transcription. Total RNA was extracted using TRIzol reagent (Lucerna Chemie AG, Luzern, Switzerland) according to the manufacturer's protocol. RNA samples were treated with DNase (Ambion, Cambridgeshire, UK) in order to remove possible contaminating genomic DNA. RNA was quantified spectrophotometrically at 260 nm (Biophotometer, Eppendorf-Vaudaux, Schönenbuch, Switzerland). The quality was assessed by gel electrophoresis on agarose gel containing ethidium bromide (EtBr, Bio-Rad Laboratories AG, Reinach, Switzerland). One microgram of total RNA was subjected to reverse transcription (RT) (Omniscript RT kit; Qiagen, Basel, Switzerland).

Polymerase chain reaction. Polymerase chain reaction (PCR) was performed on a conventional thermal cycler (TGradient, Biometra, Göttingen, Germany) using PCR Taq core kit (Qiagen). Human gene-specific, intron spanning primers were used as mentioned in Table 1 with exception of primer for Ipf-1 and insulin that were used for real-time PCR. Amplification products were visualized on agarose gels containing 0.5 μg/mL EtBr. 100 bp Molecular Ruler (Bio-Rad, Reinach, Switzerland) was run as size reference. PCR product identity was confirmed by nucleotide sequencing (Microsynth AG, Balgach, Switzerland).

Quantitative real-time polymerase chain reaction. cDNA, obtained as described above, was subjected to quantitative real-time PCR analysis using the ABI 7000 Sequence (Perkin-Elmer, USA) detection system. Specific primers yielding short PCR products suitable for Sybr-Green (Abgene, Epsom, UK) detection were designed using Primer Express software (version 2.0; PE Applied Biosystems, Foster City, CA). For sequences of primers, see Table 1. The reaction consisted of 50 μL, containing 25 μL Sybr-Green, 2 μL sense primer (10 μmol/L), 2 μL antisense primer (10 μmol/L), 16 μL H_2O, and 5 μL cDNA. Conditions were set as suggested by the manufacturer. Each cDNA sample tested for quantitative gene mRNA expression was also subjected to hypoxanthine-phosphoribosyltransferase (HPRT) mRNA analysis. Results were expressed as the ratio of the respective gene mRNA and HPRT mRNA threshold values. Gene induction in differentiated cells is expressed as percentage of values found in undifferentiated MSC. The product identity was confirmed by

Table 1
Primer sequences for RT-PCR

Gene	Sense primer	Antisense primer	Amplicon (bp)	Accession number	Cycles
HPRT	5′-TCAGGCAGTATAATCCAAAGATGGT-3′	5′-AGTCTGGCTTATATCCAACACTTCG-3′	85	M26434	40
Insulin	5′-GCAGCCTTTGTGAACCAACA-3′	5′-TTCCCCGCACACTAGGTAGAGA-3′	69	NM_000207	40
Ipf-1	5′-TGATACTGGATTGGCGTTGTTT-3′	5′-TCCCAAGGTGGAGTGCTGTAG-3′	70	NM_000209	40
ABCG2	5′-GGTTACGTGGTACAAGATGATGTTG-3′	5′-AGCCGAAGAGCTGCTGAGAA-3′	80	AY289766	40
Pax-6	5′-TGCGACATTTCCCGAATTCT-3′	5′-GATGGAGCCAGTCTCGTAATACCT-3′	81	NM_001604	40
Isl-1	5′-CAACTGGTCAATTTTTCAGAAGGA-3′	5′-TTGAGAGGACATTGATGCTACTTCAC-3′	75	NM_002202	40
Nestin	5′-CGTTGGAACAGAGGTTGGAG-3′	5′-TAAGAAAGGCTGGCACAGGT-3′	396	BC032580	40
Ngn-3	5′-CTATTCTTTTGCGCCGGTAGA-3′	5′-CTCACGGGTCACTTGGACAGT-3′	73	NM_020999	40
Somatost	5′-GATGCCCTGGAACCTGAAGA-3′	5′-CCGGGTTTGAGTTAGCAGATCT-3′	82	BC032625	40
Glucagon	5′-CCCAAGATTTTGTGCAGTGGTT-3′	5′-CAGCATGTCTCTCAAATTCATCGT-3′	80	NM_002054	40
Thy-1	5′-GTCCTTTCTCCCCCAATCTC-3′	5′-GGGAGACCTGCAAGACTGTT-3′	239	NM_033209	40
SCF	5′-GGTGGCAAATCTTCCAAAAG-3′	5′-TCTTTCACGCACTCCCACAAG-3′	222	BC074725	40
c-kit	5′-GGCATCACGGTGACTTCAAT-3′	5′-GGTTTGGGGAATGCTTCATA-3′	244	L04143	40

All primers were run at 60 °C.

sequence analysis and electrophoresis on a 3% agarose gel containing EtBr.

Peptide measurements. Somatostatin concentration was determined in culture supernatants using a commercially available somatostatin radioimmunoassay (RIA) kit (functional assay sensitivity: somatostatin 5 pg/tube, Phoenix Pharmaceuticals, Belmont, CA).

Immunocytochemistry. Cultured MSC were transferred on glass slides and incubated overnight in expansion medium. Islet-like clusters were collected after 3 days in differentiation medium and dissociated by trypsin-ethylenediaminetetraacetic acid (EDTA) in order to obtain single cells. Single cells were transferred on glass slides coated with poly-L-lysine (Sigma, Buchs, Switzerland) and incubated overnight in DMEM/F12 medium containing 10% FBS allowing them to attach. Cells were then fixed with 4% paraformaldehyde in PBS (pH 7.4) for 30 min at room temperature. After several rinses in PBS, cells were permeabilized with chilled methanol for 10 min. Unspecific binding was prevented by incubation with 10% heat-inactivated FBS in PBS at RT for 30 min. Cells were incubated with primary antisera for 60–90 min at 37 °C, rinsed off with PBS, and incubated with secondary goat antisera for 45 min at 37 °C. After several washes with PBS, cells were coverslipped with non-fluorescing mounting medium. The primary antibody rabbit anti-human c-peptide (dilution 1:500) was from Linco, St. Charles, MO. Mouse anti-Isl-1 (dilution 1:100) (number 39.4D5) was obtained from Developmental Studies Hybridoma Bank, University of Iowa, IA. The rabbit anti-human Ipf-1 (dilution 1:1000) was a generous gift from J.F. Habener, Boston, MA. The secondary antibodies from Molecular Probes (Invitrogen AG, Basel, Switzerland) were goat anti-mouse (dilution 1:1000) and goat anti-rabbit (dilution 1:1000). Goat anti-mouse IgG was labeled with Alexa fluor 488 dye. Goat anti-rabbit IgG was labeled with Alexa fluor 546 dye. 4,6-Diamidino-2-phenylindole (DAPI, 5 μg/mL) from Sigma was used to label the nuclei (10 min at 37 °C). Cells were examined by fluorescence microscope (Axiophot, Zeiss, Germany).

Results

Human adipose tissue-derived MSC from four donors were isolated and proliferated in bFGF containing culture medium. The initial cell population was positive for stem cell factor (SCF) and its receptor (c-kit). This cell population was negative for ABCG2, nestin, Thy-1, and Isl-1 as assessed by RT-PCR (Fig. 1A). Proliferating MSC, however, expressed ABCG2, nestin, Thy-1, and Isl-1 mRNA (Fig. 1A). Using immunocytochemistry we found Isl-1 protein in the nuclei of approximately 10% of cultured MSC (Fig. 1B). The mesenchymal characteristic of adipose tissue-derived MSC was described by previous studies [6,18–20] and confirmed with internal control experiments showing adoption of adipocytic and osteocytic phenotypes of these cells in response to standard protocols (data not shown).

Induction of pancreatic endocrine differentiation with our defined culture conditions was associated with stepwise decrease in the expression level of the stem cell marker ABCG2 as analyzed by quantitative real-time PCR. This was mirrored by up-regulation of Isl-1, Ipf-1, and Ngn-3 expression during the observed 3-day period (Fig. 2). Interestingly, expression of Pax-6 was also found in proliferating MSC and was not further induced by the 3-day differentiation procedure. Genes known to be positively regulated by Ipf-1 like insulin and somatostatin were also induced during the differentiation period. In addition, an activation of glucagon gene expression was observed (Fig. 3).

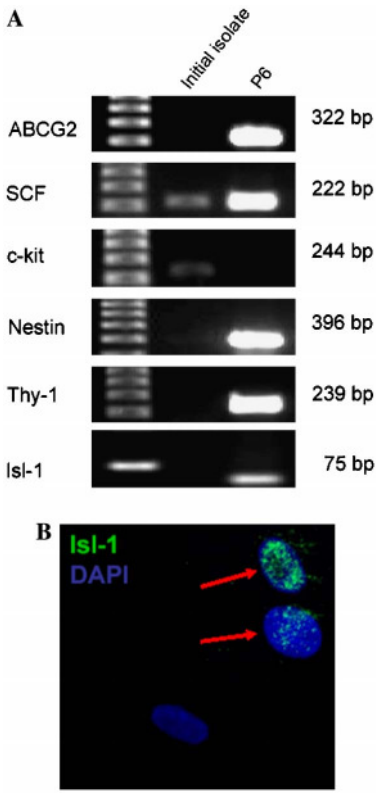

Fig. 1. Expression of stem cell markers in adipose tissue-derived MSC. (A) Presence of ABCG2, SCF, c-kit, nestin, Thy1, and Isl-1 mRNA was analyzed by RT-PCR in primary cell isolates and MSC (passage 6) in expansion medium. (B) Immunocytochemistry for the transcription factor Isl-1 (magnification 400×). Arrows indicate positive staining. Nuclei were counterstained with DAPI (in blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Nuclear staining for Ipf-1 was found in approximately 10% of the cells after 3 days (Figs. 4A and B). Ipf-1 was not detected by immunocytochemistry in undifferentiated cells. C-peptide positive cells were found in differentiated MSC (Figs. 4C and D). In addition, we found a release of somatostatin into the medium that reached plateau levels at day 3 (Fig. 4E).

Discussion

The present study demonstrates that human adipose tissue-derived MSC are able to adopt a pancreatic endocrine phenotype ex vivo. This was achieved without genetic modification and in response to defined culture conditions. Initially, these cells were cultured in high glucose (25 mM) and bFGF (5 ng/mL) and expressed after expansion not only the stem cell markers nestin, ABCG2, SCF, and Thy-1 but also the pancreatic transcription factor Isl-1 (Fig. 1). ABCG2 is a known marker for the side population phenotype stem cells in bone marrow [22] and was recently found in pancreatic islet-derived precursor cells and neural stem cells [23,24]. Together with Isl-1 it may thus point to a subpopulation of MSC with

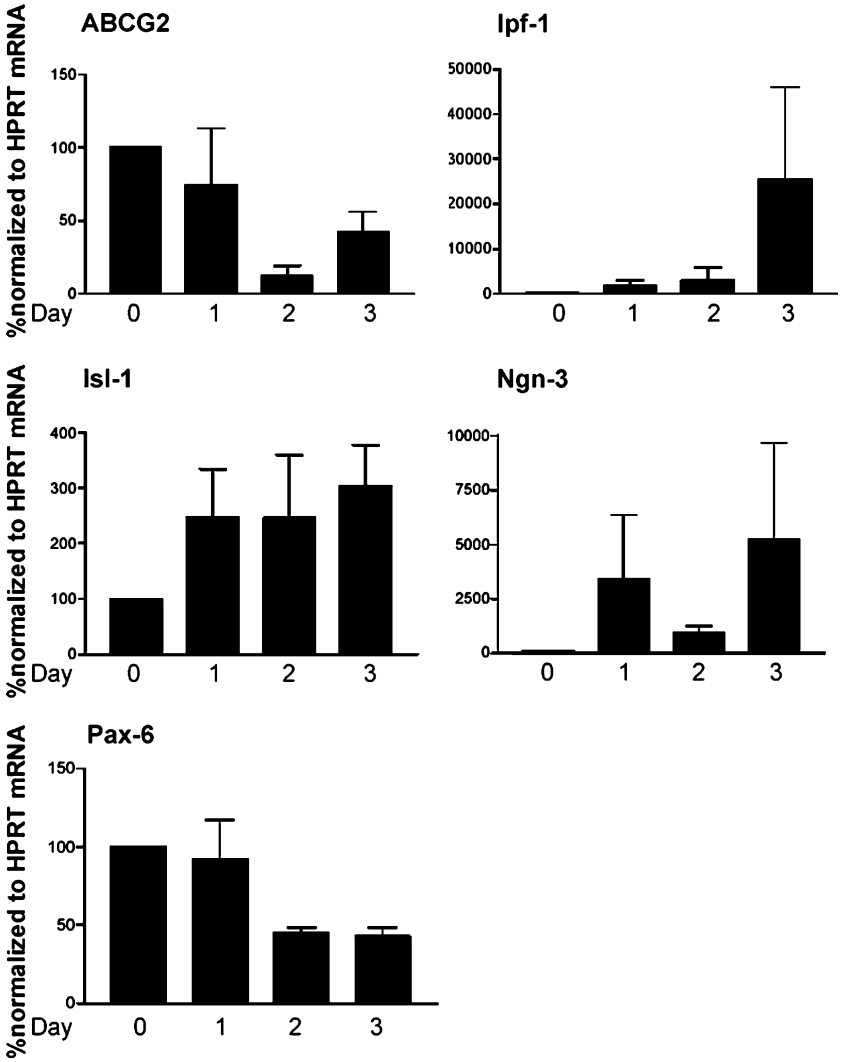

Fig. 2. Induction of pancreatic developmental genes in adipose tissue-derived MSC in response to defined culture conditions. Gene expression was monitored every 24 h for 3 days. Data are normalized to HPRT and expressed as percentage expression as compared to day 0. Results are means ± SEM of quadruplicate of four independent experiments from four independent donors. mRNA expression of pancreatic transcription factors Isl-1, Ipf-1, Ngn-3, and the side population marker ABCG2 and Pax-6 was analyzed by real-time PCR.

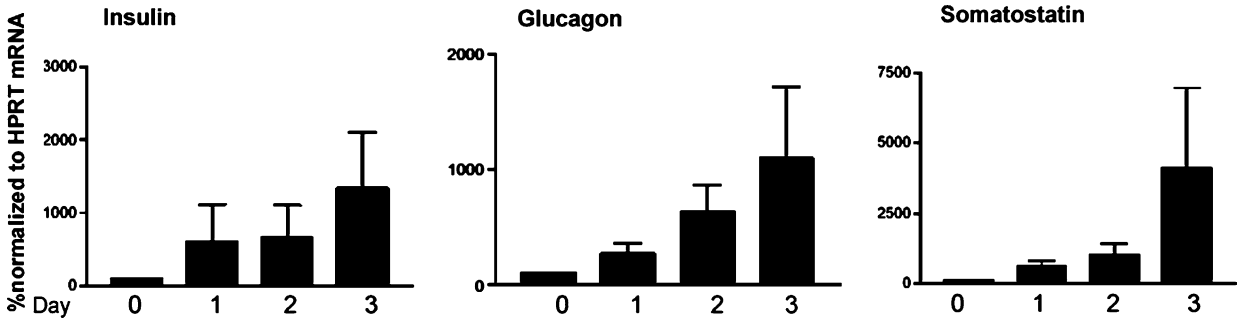

Fig. 3. Induction of the islet genes insulin, glucagon, and somatostatin in adipose tissue-derived MSC in response to defined culture conditions.

neuro-endocrine developmental potential. The transcription factor Isl-1 is crucial for the development of pancreatic endocrine cells. Disruption of Isl-1 expression is associated with absence of dorsal mesenchyme and a marked reduction of Ipf-1 gene expression in dorsal epithelium in mice [25]. Embryonic explants of the pancreatic anlage from Isl-1 (−/−) mice did not generate insulin, glucagon or somatostatin positive cells in vitro as did explants from Isl-1 (+/−) animals [25]. Isl-1 expression is together with Ipf-1 one of the earliest pancreatic transcription factors detected during development [25]. Induction of Isl-1 expression in our human MSC

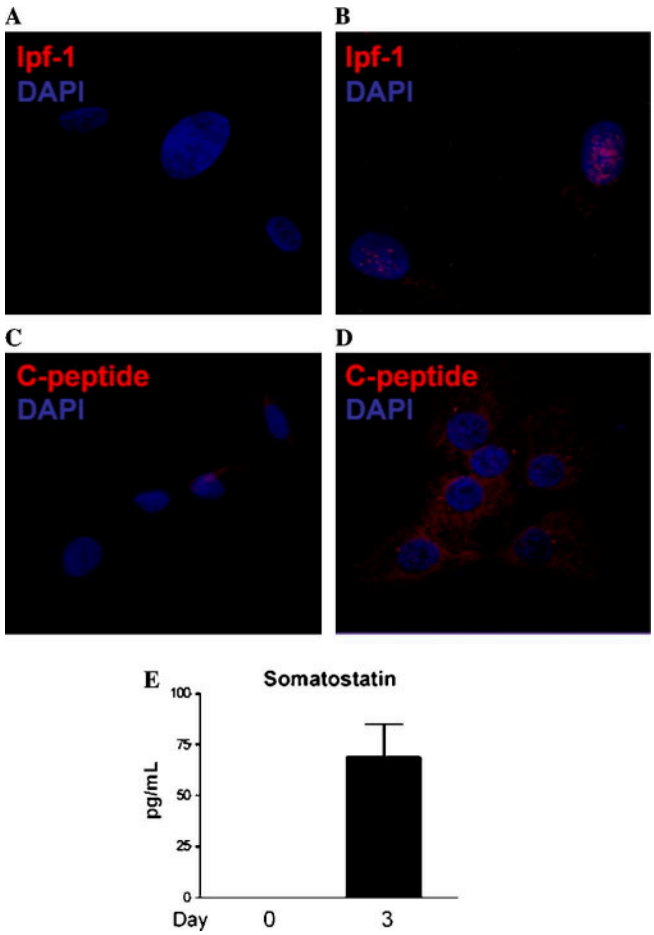

Fig. 4. Immunocytochemistry for Ipf-1 and C-peptide in undifferentiated and differentiated adipose tissue-derived MSC. Single cells were stained for Ipf-1 (A and B, magnification 400×) and c-peptide (C and D, magnification 200×). Undifferentiated MSC were used as negative control (A and C). Nuclear staining was performed in blue with DAPI. (E) Somatostatin release during the differentiation period was measured with RIA in supernatants collected on day 0 and 3 ($n = 8$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

may represent a critical event that allows adoption of a pancreatic endocrine phenotype. Other early markers of pancreas development like Ipf-1 and Ngn-3 were not detected by PCR in undifferentiated cells.

Using serum-free medium supplemented with factors known for their beneficial effects on differentiation of precursor cells into insulin producing cells (i.e., exendin-4, pentagastrin, activin-A, betacellulin, nicotinamide, and hepatocyte growth factor) [26–29] we have induced an activation of pancreatic transcription factors including Ipf-1, Isl-1, Ngn-3, as well as the islet proteins insulin, glucagon, and somatostatin. The real-time PCR studies revealed an activation of some of these pancreatic genes already 24 h after induction of differentiation (Figs. 2 and 3). Similar early induction of insulin gene expression was reported recently in mice in vivo in response to hyperglycemia [30]. Here, insulin positive cells were identified in adipose tissue, spleen, and also bone marrow of hyperglycemic mice after 3 days.

Several studies addressed the possibility that bone marrow-derived stem cells could contribute to β-cell turnover in vivo. Some of them presented positive results, suggesting a circulating pool of stem cells that could participate in the process of β-cell neogenesis [31]. These results, however, were not confirmed by others [32] and a recent report questioned the entire concept of β-cell stem/progenitor cells with studies using genetic lineage tracing experiments [33]. With this approach it has been shown that pre-existing β-cells rather than adult stem/progenitor cells retained a proliferative capacity and may thus represent the major source of new β-cells in adult life, at least in mice [33]. While in vivo studies are not conclusive, this does not exclude the differentiation of adult stem cells into insulin producing cells in vitro. It has been shown that mouse mesenchymal stem cells from bone marrow cultured in high glucose over 4 months induced several β-cell-specific genes including insulin and Ipf-1 [16]. These cells were also able to reverse hyperglycemia in an animal model of diabetes [16].

In summary, Isl-1 positive MSC can be isolated from human adipose tissue and are able to adopt a pancreatic endocrine phenotype. These cells could be used as a human model to develop stem cell-based therapies for diabetes mellitus.

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7. APPENDIX

7.1. Acknowledgements

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1987 – 1995	Realgymnasium in Basel, Matura typus B (latin).

Publications

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von Mach MA, Hengstler JG, Marc Brulport M, **Eberhardt M**, Schormann W, Hermes M, Prawitt D, Zabel B, Grosche J, Reichenbach A, Müller B, Weilemann LS and Zulewski H. *"In vitro cultured islet-derived progenitor cells of human origin express human albumin in SCID mouse liver in vivo"*. **Stem Cells** 2004; 22: 1134 – 1141.

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Timper K*, Seboek D*, **Eberhardt M**, Linscheid P, Christ-Crain M, Keller U, Müller B and Zulewski H. *"Human adipose tissue-derived mesenchymal stem cells differentiate into insulin, somatostatin and glucagon expressing cells"*. **Biochem Biophys Res Commun** 2006; 341: 1135 – 1140.

Seboek D*, Timper K*, **Eberhardt M**, Linscheid P, Keller U, Martin I, Barbero A, Müller B and Zulewski H. *"Human mesenchymal stem cells from non-diabetic and type 1 diabetic patients differentiate into insulin, somatostatin and glucagon expressing cells"*. In preparation.

* **NB**: authors equally contributed to the study

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Oral presentations at:

- The 12th International Congress of Endocrinology 2004, Lisbon, Portugal
- Schweiz. Gesellschaft für Endokrinologie und Diabetologie 2004, Bern, Switzerland
- The 41st European Association for the Study of Diabetes 2005, Athens, Greece

Poster presentations at:

- The American Endocrine Society's 85th Annual Meeting 2003, Philadelphia, USA
- The American Endocrine Society's 86th Annual Meeting 2004, New Orleans, USA
- Schweiz. Gesellschaft für Endokrinologie und Diabetologie 2005, Bern, Switzerland

During the PhD-Program at the University of Basel I have attended the following lectures:

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7.3. Declaration

I declare that I wrote this thesis

ISOLATION AND CHARACTERIZATION OF MESENCHYMAL STEM CELLS FROM CULTURED HUMAN PANCREATIC ISLETS OF LANGERHANS

with the help indicated and only handed it in to the faculty of science of the University of Basel and to no other faculty and no other university.

Basel, 2006

Michael Eberhardt