Differentiation of embryonic stem cells into pancreatic insulin-producing cells

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1. Introduction

Lack or defect of insulin-producing pancreatic beta cells results in diabetes, a devastating disease suffered by 150 million people worldwide. Most of diabetic patients require exogenous insulin injections, that delay but do not prevent from long-term complications. Pancreatic beta cells show a low regeneration capacity, therefore cell replacement strategies are considered as promising approaches in the treatment of diabetes. Because the number of immunologically compatible human donor tissues is limited, a wide scale application of pancreas or pancreatic islets transplantations is not possible. Stem cells of embryonic and adult origin (Fig. 1) represent an attractive cell source for the generation of a sufficient amount of beta cells that could be transplanted to diabetic people.

Stem cells are defined by their ability to both, self-renew and differentiate into specialized cells [see rev. (Czyz et al., 2003)]. Embryonic stem (ES) cells are characterized by nearly unlimited proliferation and the capacity to differentiate into derivatives of nearly all lineages. Pluripotent ES cells represent potentially unlimited source of pancreatic cells for regenerative therapies. However, so far, current techniques do not allow the generation of pure populations of somatic cells from ES cells.

The presence of adult stem cells in a wide range of tissues gives an opportunity to employ autologous stem cells for the generation of immunologically compatible transplantable cells. Adult stem cells regenerate mainly the effector cells of their own tissue. The plasticity or "transdifferentiation" potential of adult stem cells is controversial and still under debate (Wagers and Weissman, 2004). In contrast to ES cells, adult stem cells have no tumorigenic potential and could be used in autologous transplantations. However, problems related to low proliferation and the limited developmental capacity create barriers for the therapeutic application of adult stem cells in regenerative medicine. Therefore, studies with both, ES cells and adult stem/progenitor cells are required, because knowledge and experience

from one stem cell system may be extrapolated to the other and finally will result in applicable cell therapies.

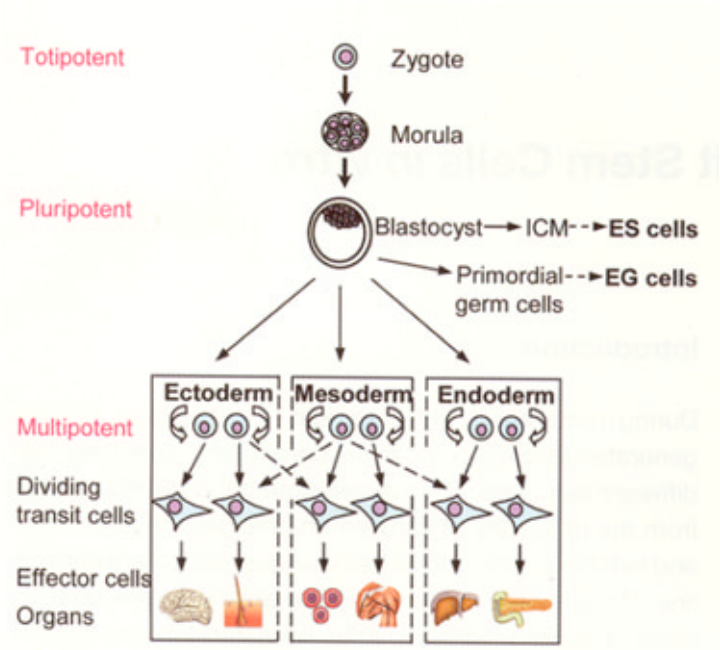

Fig. 1. Hierarchy of stem cell plasticity (according to Czyz et al., 2003)

2. Aim of the study

The main aim of this study was (i) to develop a cultivation strategy suitable for the generation of functional insulin-producing cells from ES cells *in vitro*. Secondly, our studies were focused (ii) to investigate the influence of constitutive expression of genes involved in beta cell development, specifically of Pax4 and Pdx1, on pancreatic ES cell-derived differentiation. Moreover, our aim (iii) was to identify potential pancreatic progenitor cells involved in the differentiation of ES cells into the pancreatic lineage and to characterize mechanisms and processes of islet-like cluster formation *in vitro*.

3. Development and function of pancreatic beta cells

3.1. Pancreas organogenesis

The pancreas is an organ containing two different types of tissue: the exocrine cells that secrete enzymes into the digestive tract, and the endocrine cells that secrete hormones into the bloodstream. The functional unit of endocrine pancreas is the islet of Langerhans, which is composed of four cell types: alpha, beta, delta and PP cells that produce glucagon, insulin, somatostatin and pancreatic polypeptide, respectively and form spheroidal clusters embedded to the exocrine tissue.

Pancreas arises from the endoderm as a dorsal and a ventral bud which fuse together to form the single organ (Slack, 1995). Specification of the pancreas region in mouse begins at embryonic day (E)7.5 of development, when signals from mesoderm and ectoderm establish the anterior-posterior pattern of the endoderm (Wells and Melton, 2000). At the E8.5 of mouse development the notochord separates the neural tube and the gut endoderm. One of the earliest detected event in pancreas development is the repression of Sonic hedgehog (Shh) by signals from the notochord, such as activin-betaB and FGF-2, which promote expression of a homeobox transcription factor Pdx1 (known also as Ipf-1, Idx-1 or Stf-1) in the adjacent pancreatic epithelium (Hebrok et al., 2000). Additionally, the repression of Shh is important in determining the differentiation of the surrounding mesoderm into specialized intestinal or pancreatic mesenchyme. At E9.5, dorsal aorta displace notochord and initiate pancreatic budding. Further, the mesenchyme separates pancreatic epithelium from dorsal aorta. Signals from the surrounding mesodermic tissue, such as follistatin and VEGF-A regulate expression of transcription factors in the pancreatic epithelium and are responsible for specification of endocrine versus exocrine tissues (Miralles et al., 1998; Lammert et al., 2003).

Once dorsal and ventral pancreatic buds develop, the undifferentiated pancreatic epithelium is characterized by the expression of several transcription factors, such as: Hlxb9, Hnf6, Hnf3beta and Pdx1. Hlxb9 is required for the initial pancreatic budding (Harrison et al., 1999). Hnf6 induces Hnf3beta expression that is a transcriptional regulator of Pdx1 (Jacquemin et al., 2000; Wu et al., 1997). Pdx1 is broadly expressed in the pancreatic

epithelium and also in the adjacent duodenum and antral stomach till E13.5, when its expression becomes restricted to most of beta and some delta cells. However low expression of Pdx1 is detectable in some ductual and exocrine cells (Ohlsson et al., 1993). Pdx1 mutant mice do not develop any pancreas, and the pancreatic development is arrested after initial bud formation (Jonsson et al., 1994; Ahlgren et al., 1996; Offield et al., 1996). It demonstrates that Pdx1 is necessary for the growth of the pancreatic buds but not for the initial induction of bud formation. In adult organism, Pdx1 is involved in the regulation of expression of pancreatic genes including *insulin* (Ohlsson et al., 1993), *somatostatin* (Leonard et al., 1993), *glucose transporter 2* [Glut2 (Waeber et al., 1996)], *glucokinase* (Watada et al., 1996) and *islet amyloid polypeptide* [IAPP (Macfarlane et al., 2000)].

Early pancreatic precursors expressing uniformly Pdx1 and other factors differentiate into mature islets and acinar cells. The specification of endocrine cells in the developing pancreatic endoderm is regulated by the Notch signalling pathway, a mechanism involved also in the specification of neurons in the developing neuroectoderm. During neural development, expression of basic helix-loop-helix (bHLH) transcription factors of the *neurogenin* gene family leads to the development of neural precursor cells and in parallel, activation of Notch receptor on adjacent cells results in the repression of neurogenin (and other target genes) expression, thereby preventing neuronal differentiation in cells adjacent to developing neuroblasts (Anderson et al., 1997; Baker, 2000). In the developing pancreatic epithelium, individual cells or small cell clusters express neurogenin 3 (ngn3), a member of the *neurogenin* gene family. Ngn3 is expressed only in progenitor cells before islet formation and is undetectable in adult pancreas (Apelqvist et al., 1997; Schwitzgebel et al., 2000; Gradwohl et al., 2000). Animals deficient for ngn3 fail to develop any endocrine cells (Gradwohl et al., 2000), whereas uniform ectopic expression in the pancreatic epithelium results in massive premature differentiation of the entire pancreas into endocrine cells (Apelqvist et al., 1997; Schwitzgebel et al., 2000). Hnf6 was shown to regulate ngn3

expression. Hnf6 null mice have reduced ngn3 expression in the developing pancreas and reduced number of endocrine cells that were not organized into islets (Jacquemin et al., 2000).

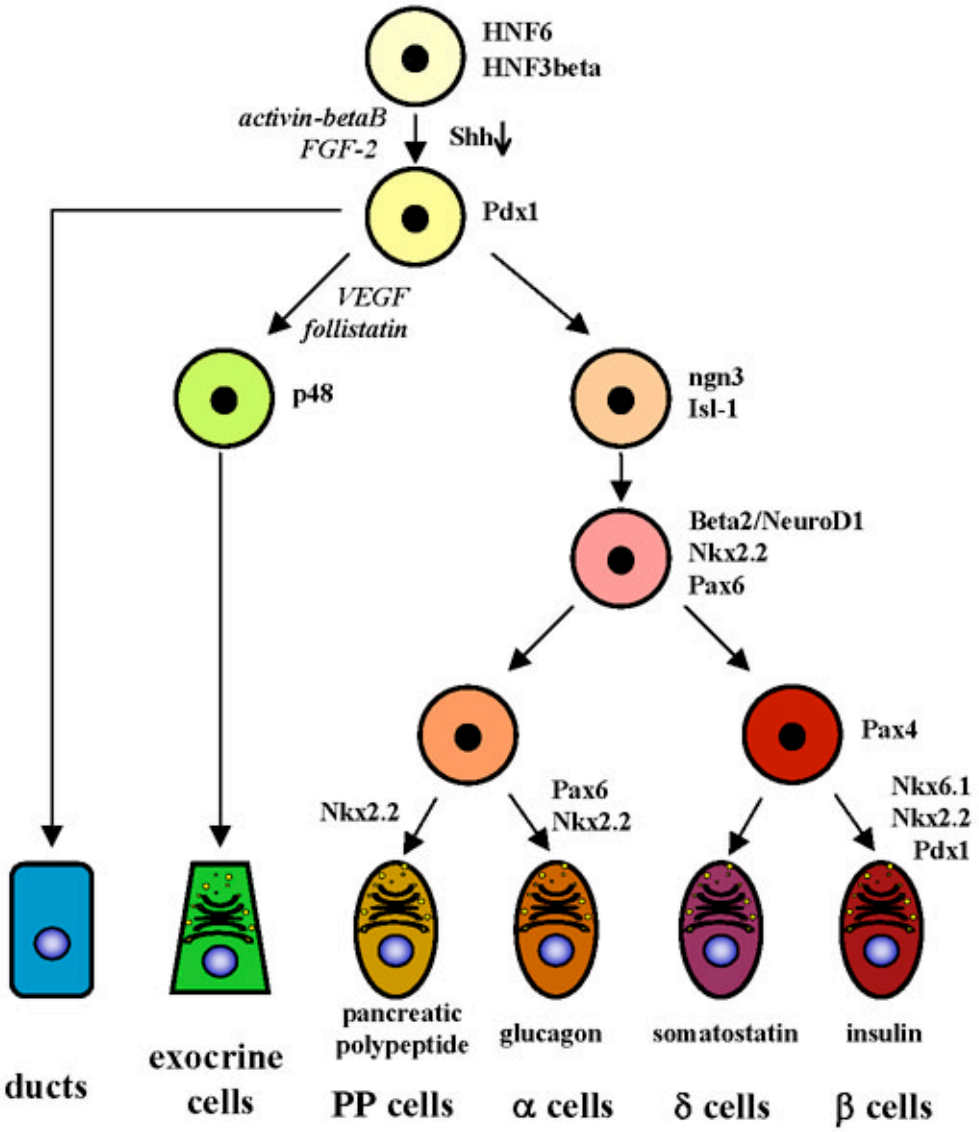

Fig. 2. A simplified model of pancreatic development summarizing involvement of signalling molecules (italic) and transcription factors

Beta2/NeuroD1 and p48 represent another bHLH transcription factors involved in pancreatic development. Expression of Beta2/NeuroD1 is detected slightly after ngn3, however in contrast to ngn3, Beta2/NeuroD1 is expressed in mature islets, where it plays a role in the expression of different products of endocrine cells including insulin (Naya et al., 1995; Glick et al., 2000). Expression of Beta2/NeuroD1 is lost in ngn3 deficient mice (Gradwohl et al., 2000), whereas in Beta2/NeuroD1 null mice, ngn3 expression is unchanged,

but the number of pancreatic islets is strongly reduced due to accelerated apoptosis (Schwitzgebel et al., 2000; Naya et al., 1995). These data suggest that Beta2/NeuroD1 is located downstream of ngn3.

In contrast to Beta2/NeuroD1 that is involved in endocrine development, p48 is required to drive cells into the exocrine lineage. In the absence of p48, pancreatic exocrine tissue fails to develop (Krapp et al., 1998).

A number of transcription factors including Isl-1, Pax4, Pax6, Nkx2.2 and Nkx6.1 were identified to be expressed during development of endocrine lineages. These transcription factors might play a role in endocrine cell subtype fate decision, however until now convincing data are still missing.

The LIM homeodomain factor Isl-1 is required for the generation of all endocrine cells. Animals lacking Isl-1 have no endocrine cells indicating a function of Isl-1 in the generation of endocrine progenitor cells. Moreover, Isl-1 is also required for exocrine cell development in the dorsal bud (Ahlgren et al., 1997).

Pax4 and Pax6 belong to the paired-homeodomain transcription factor family of *Pax* genes that are involved in the formation of many organs (Dohrmann et al., 2000). During pancreas development, Pax6 is restricted to the endocrine lineage and its expression is maintained in endocrine cells in adults (Sander et al., 1997; St Onge et al., 1997). Pax6 is specifically involved in alpha cell development. Pax6 knock-out animals do not form glucagon-producing alpha cells and the morphology of islets is disrupted (St Onge et al., 1997). There is further evidence that Pax6 is not only required for alpha-cell differentiation, but is also involved in the proliferation of all endocrine cells (Sander et al., 1997).

In contrast to the widespread embryonic expression of Pax6, Pax4 is characterized by an unique expression pattern restricted to the endocrine pancreas and to few cells in the ventral spinal cord (Sosa-Pineda et al., 1997). Pax4 expression is detected during embryogenesis beginning at E10.5 with maximal expression at E15.5, followed by continuous decrease and is

undetectable in adult pancreas (Dohrmann et al., 2000). Animals deficient for Pax4 completely lack cells of the beta- and delta-cell lineages, whereas the number of alpha cells is significantly increased (Sosa-Pineda et al., 1997). These findings suggest that after development of endocrine progenitors, cells expressing Pax4 become more restricted to the insulin- and somatostatin-cell fate. Despite of the critical role of Pax4 expression in the pancreatic beta- and delta-cell development, ectopic Pax4 expression is insufficient to drive ngn3-positive precursors into beta- and delta-cell fate (Grapin-Botton et al., 2001). Instead, Pax4 is a direct target of ngn3, because ngn3 in cooperation with Hnf1alpha was reported to bind and activate Pax4 gene promoter (Smith et al., 2000; Smith et al., 2003). Moreover, in ngn3 null mutants, Pax4 expression is lost in the pancreas (Gradwohl et al., 2000).

Two other transcription factors involved in pancreatic endocrine development are Nkx2.2 and Nkx6.1 that belong to the *NK homeodomain* gene family. Nkx2.2 is broadly expressed in the pancreatic bud and after E13.5, its expression becomes restricted to ngn3-positive progenitors, however in contrast to ngn3, the expression of Nkx2.2 is maintained in mature endocrine cells. Mice lacking Nkx2.2 have a complete absence of beta cells and a reduced number of alpha and PP cells. The mutant animals develop islets that contain alpha, delta, PP cells and a cell population with abnormal characteristics of beta cells expressing Pdx1 and IAPP, but not Glut2 and glucokinase (Sussel et al., 1998). These results suggest that in the absence of Nkx2.2, beta cells are specified but unable to maturate into functional beta cells.

The expression pattern of Nkx6.1 during embryogenesis and in adults is similar to Nkx2.2 with the exception that Nkx6.1 is not expressed in non-beta islet cells (Oster et al., 1998; Sander et al., 2000). Mice lacking Nkx6.1 have defects in beta cell generation, but in contrast to Pax4 mutants, delta cells are unaffected (Sander et al., 2000). There are evidences that Nkx2.2 lies upstream of Nkx6.1 and regulate its expression during beta cell development. Nkx2.2 expression is unaffected in Nkx6.1 null mutants, whereas in mice lacking Nkx2.2,

Nkx6.1 is absent (Sussel et al., 1998; Sander et al., 2000). Additional studies showed that the Nkx6.1 promoter can be regulated by Nkx2.2 (Watada et al., 2000). A schematic representation of pancreas development is shown on Fig. 1.

3.2. Function of pancreatic beta cells

The main function of pancreatic beta cells is the production and controlled release of insulin. Insulin is a hormone composed of two polypeptide chains A and B linked by two disulphide bonds. Insulin is synthesized as a single-chain preproinsulin composed of two A and B chains, connecting peptide (C-peptide) and signalling peptide. C-peptide joins the carboxyl end of the B chain and the amino terminus of the A chain (Fig 3). Preproinsulin is converted in the endoplasmic reticulum into proinsulin, that is transported to the Golgi complex and then to secretory granules, where the connecting peptide is proteolyzed (see Fig. 3). Insulin molecules in storage granules are secreted when the membrane of a granule fuses with the plasma membrane of the cell.

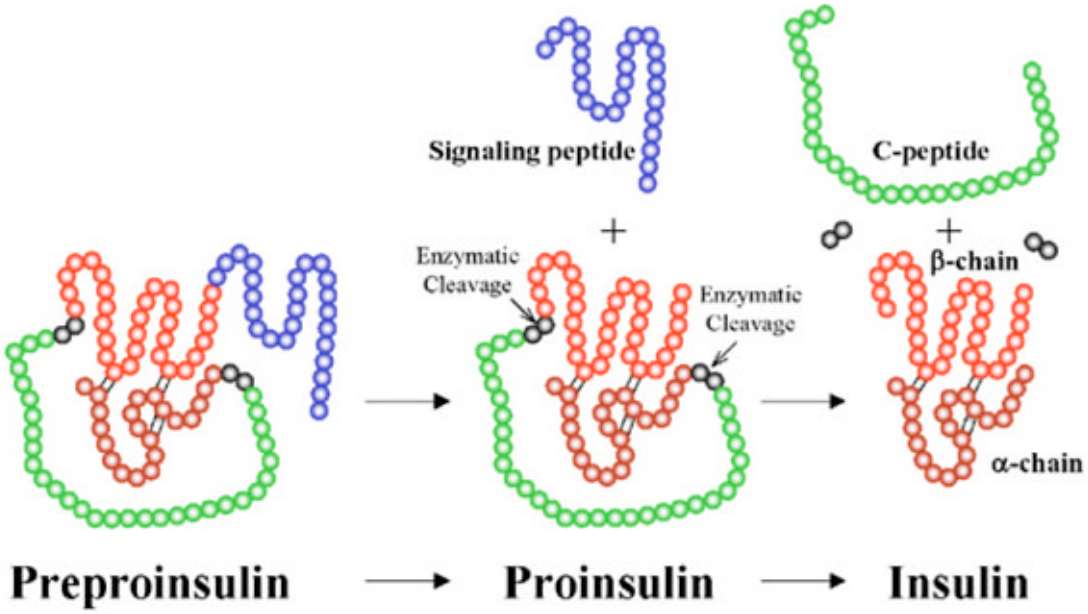

Fig. 3. Schematic representation of conversion of preproinsulin into insulin

The function of insulin in the organism is stimulation of glucose uptake from the blood and its storage in cells. Insulin activity affects muscle, liver and fat cells. In muscle cells, insulin increases glucose uptake and stimulates its conversion into glycogen. In hepatocytes, insulin prevents the breakdown of stored glycogen (glycogenolysis) and the synthesis of new glucose (gluconeogenesis). In lipid cells, insulin promotes conversion of glucose into glycerol, that further forms triglycerids and prevents fat breakdown (lipolysis). The main inducer of insulin release is a high glucose concentration (above 10 mM), however insulin release is also induced by other factors, such as high amino acid and fatty acid levels in the blood, hormones released from the stomach and intestine as well as neurotransmitters (Lang, 1999). Entry of glucose into pancreatic beta cell and its further metabolism in mitochondria alters the adenosine triphosphate (ATP)/ adenosine diphosphate (ADP) ratio that leads to closure of ATP-sensitive K^+ (K_{ATP}) channels. It results in membrane depolarisation ($\Delta\Phi$) and opening of voltage-dependent calcium channels (VDCC). The subsequent increase in cytosolic free Ca^{+2} coupled with the multiple phosphorylation events modulated by protein kinase C (PKC) and protein kinase A (PKA) induce exocytosis and insulin secretion [(Ashcroft et al., 1994), see Fig. 4]. Insulin secretion is further regulated by several hormones and neurotransmitters. Acetylocholine (ACh) and cholecystokinin (Cck) promote phosphoinositide breakdown with a consequent mobilisation of Ca^{+2} from intracellular stores leading to activation of PKC. Other factors including glucagon-like peptide 1 (GLP-1) or glucose-dependent insulinotropic peptide (GIP) raise cyclic AMP (cAMP) levels and activate PKA. Insulin secretion can also be regulated by chemical compounds. Tolbutamide is a sulphonylurea inhibitor that inactivates the K_{ATP} channels, thus inducing insulin secretion even at low glucose concentration, whereas nifedipine is a blocker of Ca^{+2} channels resulting in inhibition of insulin release at inducible glucose concentration [(Henquin, 2000), see Fig.4].

Insulin is the only hormone that reduces blood glucose level, in contrast to a number of hormones that can raise blood glucose levels, such as glucagon, cortisol, growth hormone, thyroid hormone and adrenaline.

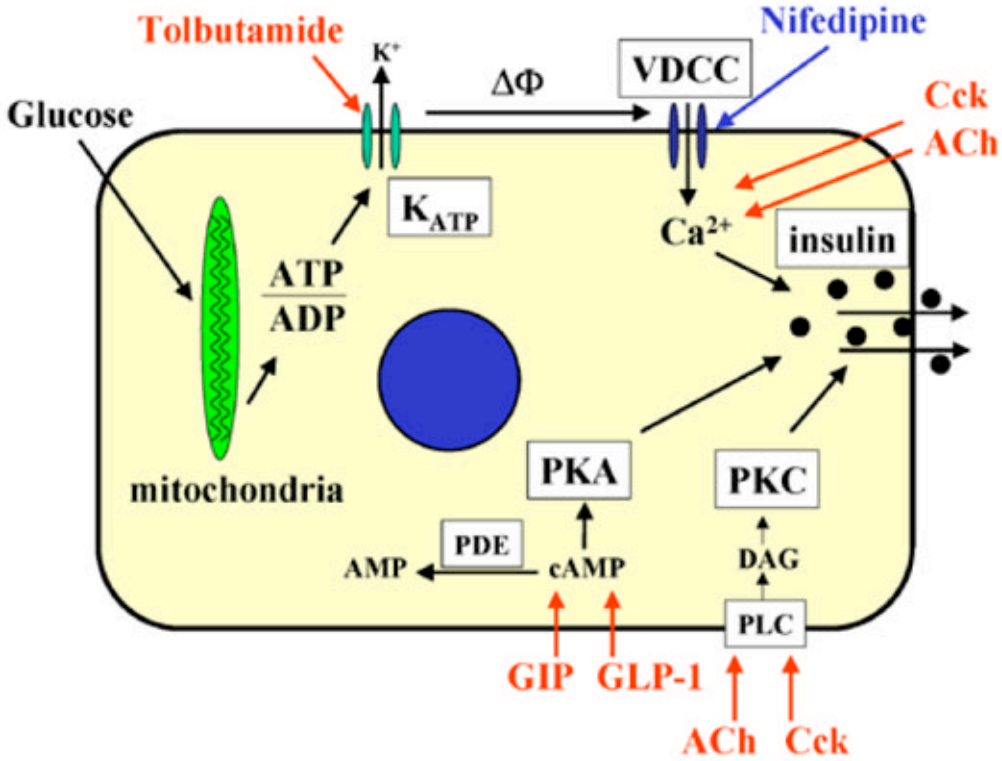

Fig. 4. Schematic representation of regulation of insulin secretion in response to glucose, hormones and pharmacological regulators. Substances indicated in red induce insulin secretion, whereas substances indicated in blue repress insulin secretion.

4. Diabetes

4.1. Types of diabetes

Diabetes, hyperglycaemia and impaired glucose tolerance are endocrine disorders characterized by inadequate production or use of insulin resulting in abnormal levels of glucose in the blood. Chronic hyperglycaemia is thought to lead to the formation of high levels of highly reactive advanced glycation endproducts (Feldman et al., 1997), that are responsible for most of the complications in diabetes including blindness, kidney failure, cardiovascular diseases, stroke, neuropathy and vascular dysfunctions. Diabetes can be

classified into two major groups: type 1 diabetes also known as insulin-dependent diabetes mellitus (IDDM), characterized by autoimmune destruction of insulin-producing beta cells, and type 2 diabetes also known as non-insulin-dependent diabetes mellitus (NIDDM), characterized by insulin resistance and impaired glucose tolerance, where insulin is not efficiently used or is produced in insufficient amounts.

For patients with type I and type II diabetes, the main treatment is based on the injection of exogenous insulin or insulin analogues. Hope for the treatment of diabetes are gene and cell replacement therapies, however these strategies require significant improvements for wide scale clinical applications. Before new therapies become available, diabetes patients require insulin injections. The development of new forms of insulin with more rapid onset or longer duration of action (insulin analogues) and new forms of delivery (continuous pumps and aerosol sprays), as well as advances in glucose level monitoring, provide other options for the diabetes patients.

4.2. Treatment of type I diabetes

Type I diabetes is a disease characterised by a destruction of insulin producing beta cells by the body's own immune system. The gene thought to be responsible for this reaction is located within a region of the major histocompatibility complex (MHC) HLA class 2 gene, called IDDMI. The role of this gene in the immune response is presentation of foreign molecules to T cells, however the detailed action in type I diabetes is still under investigation. Therefore, the treatment of patients with type I diabetes is currently limited to insulin injections.

The commonly available human insulins represent groups of short-, intermediate- and long-acting insulins and biphasic mixtures. All groups have different characteristics with respect to the onset and duration of action. DNA recombination technologies provide the opportunity for the creation of insulin analogues with improved function. One group

represents rapid-acting analogues known also as “rapid-onset” and “ultra-short-acting” insulins and include *insulin aspart* and *insulin lispro*. Both recombinants contain modifications in the amino acid sequence of the insulin B chain. The advantage of using these analogues is a faster response in comparison to conventional short-acting insulin, therefore they can be injected immediately before meals or even after eating and are very useful for patients of young age. The disadvantage of the rapid-acting analogues is the relatively short duration of insulin action. Another group of recombinant insulins are long-acting analogues that include *insulin glargine* (created by substitution and adding amino acids) and *insulin detemir* (created by adding a fatty acid chain to enhance binding to albumin). The advantage of the long-acting analogues is a more reproducible absorption and reduced risk of hypoglycaemia (UBS Warburg Report, 2001).

4.3. Treatment of type II diabetes

In contrast to type I, development of type II diabetes is a gradual process. One of the earliest symptoms is insulin resistance, the impairment of the ability of the body to respond to insulin. Insulin resistance leads in early stages of type II diabetes to increased insulin secretion, however glucose tolerance remains normal. Different mechanisms including problems related to the insulin receptor and signal transduction mechanisms, changes in the insulin target protein levels and interference from other hormonal systems finally induce the disease. However, the primary cause seems to be the accumulation of lipids in the muscles and liver. Over time beta cells are not able to maintain the high rate of insulin production and become exhausted. This leads to impaired glucose tolerance, an intermediate state between normal glycaemic control and uncontrolled blood glucose levels. Patients with impaired glucose tolerance have higher blood glucose levels than healthy people, but do not reach the diagnostic criteria of diabetics.

Type II diabetes patients have a wider range of treatment options in comparison to type I diabetics. Whereas, type I diabetes is currently treated only with insulin application, for

treatment of type II diabetes additionally a lifestyle modification and the application of oral hypoglycaemic agents can be employed. Diet and exercises help type II diabetics to control their blood glucose, blood pressure and blood lipid levels.

Five groups of oral hypoglycaemic agents are currently used in treatment of type II diabetes: alpha-glucosidase inhibitors, sulphonylureas, prandial glucose regulators, glitazones (thiazolidinediones) and biguanides (UBS Warburg Report, 2001). Often, the combination of different anti-diabetic drugs are used to increase the efficiency of therapy.

Alpha-glucosidase inhibitors act by inhibition of glucose absorption from intestine. They are commonly used in combination with diet and exercises. Sulphonylureas and prandial glucose regulators act via ATP-dependent potassium channels and result in release of endogenous insulin from beta cells. Sulphonylureas directly block ATP-dependent potassium channels (Trube et al., 1986), whereas prandials affect other receptors, however inactivate the same ATP-dependent potassium channels. Prandials are additionally characterized by short onset and duration of action (Owens, 1998). Therapy with sulphonylureas and prandials can be used only at the time, when beta cells become exhausted and are not able to produce insulin. These therapies may also cause hypoglycaemia when improperly used. Another class of anti-diabetic agents are glitazones that stimulate glucose uptake in muscle and liver via activation of Peroxisome Proliferator Activated Receptor-gamma (PPAR-gamma). Disadvantage of using glitazones are their toxic effects on liver and weight gain, because PPAR-gamma is involved in lipid metabolism and in the creation of new fat cells (Kim and Ahn, 2004). The fifth group of anti-diabetic agents are biguanides that in contrast to other groups affect various physiological effects in different organs and have complex mechanism of action. Results of their action is inhibition of glycolysis and gluconeogenesis in the liver, reduction of glucose absorption in intestine and increase of glucose sensitivity, uptake and utilization in the muscle. Because of indirect mechanisms, biguanides cause less side effects and are allowed to be used in combination with other anti-diabetic drugs.

5. Future strategies for the treatment of diabetes

5.1. Drugs

All substances described represent drugs that are available on the market. In this paragraph, novel peptides that are under development for treatment of type I and type II diabetes are presented. The most important molecule is glucagon-like peptide 1 (GLP-1), a peptide produced in L cells located in the intestine (Drucker, 1998). GLP-1 stimulates nutrient- or sulfonylureas-induced insulin release, stimulates insulin synthesis, increases beta cell mass and suppresses glucagon secretion from alpha cells (Drucker, 1998; Perfetti et al., 2000). Clinical studies on type II diabetes have shown that subcutaneous injection with GLP-1 normalized glycaemia in poorly controlled diabetics by stimulating beta cells function, suppressing glucagon secretion and gastric emptying. Agonists of PPAR-alpha represent another class of new drugs. PPAR-alpha belongs to the same family of nuclear factors as PPAR-gamma and is also involved in lipid metabolism. Agonists of both, PPAR-alpha and PPAR-gamma lowers lipids levels, however by different mechanisms (Sugden and Holness, 2004). Combination of PPAR-alpha/gamma agonists may have an attractive effect on lipid and glucose levels. Another hormone secreted by pancreatic beta cells is amylin, that may contribute to normal glucose regulation. Pramlintide, an amylin analogue was reported to improve glucose and weight control in insulin-treated patients with type II diabetes (Ratner et al., 2002). Altogether, amylin analogues were considered as an option in type II diabetes treatment, however gastrointestinal-related and hypoglycaemia side effects have still to be solved.

5.2. Gene therapies

Development of gene transfer technologies provided new perspectives for diabetes therapies. These include different approaches based on genetic modification of cells resulting in the delay or prevention of the onset of diabetes. As mentioned above, type I diabetes is characterized by an autoimmune destruction of beta cells. The genetic modification of beta cells or of the immune system could prevent autoimmune destruction of beta cells or result in islet regeneration.

Because, the reason for development of type I diabetes is a failure of the immune system in tolerating beta cells, therefore approaches based on tolerance induction by elimination of T cells that recognize islet or beta cell antigens might prevent the immune destruction of beta cells. Transgenic expression of the proinsulin gene controlled by the MHC class II promoter resulted in expression of proinsulin in thymus and elimination of proinsulin-reactive T cells, leading to the prevention of type I diabetes in an animal model (French et al., 1997). Injection of islets or putative autoantigens such as insulin B-chain or GAD65 proteins in thymus also suppressed the onset of type I diabetes (Posselt et al., 1991; Cetkovic-Cvrlje et al., 1997). Injection of autoantigens into thymus of people with high genetic risk for developing type I diabetes is one possible therapy strategy, however more clinical trials are necessary to verify the efficiency of this strategy.

Another strategy to preserve immunologic damage of islets is focused on the function of the antigen presenting cells (APC). The role of APC in response to local injury is uptake and presentation of antigens to T cells that induce immune response, therefore APC residing in pancreas were considered as a target for gene therapy in type I diabetes. Genetic modification of APC in pancreas allows for local production of molecules, that may interfere with activation and processing of antigens by APC, limit upregulation of adhesion molecules or limit interaction of APC-secreted factors with target cells. Approaches included the use of interleukin-1 beta receptor antagonist protein (Giannoukakis et al., 1999), CTLA4-Ig [soluble

fusion protein of human cytoxic T-lymphocyte associated protein 4 and the immunoglobulin G1 Fc region (Lenschow et al., 1992)], the soluble form of CD40 (Kenyon et al., 1999) and Fas ligand (Swenson et al., 1998).

Pancreatic beta cells or whole islets were also considered as targets for gene transfer technology. Expression of the T helper 2 cytokine IL-4 in pancreatic beta cells was shown to prevent the development of autoimmunity in non-obese diabetic mice (Mueller et al., 1996). Another successful approach that prevented autoimmune diabetes in animal model relied on modification of class I MHC expression in islets mediated by the gp19 glycoprotein from the adenoviral E3 cassette (von Herrath et al., 1997).

Gene transfer technologies were also employed for the optimisation of insulin release from existing beta or non-beta cells. As described above, GLP-1 is a hormone produced in gut characterized by insulinotropic activity. Implantation of the encapsulated (in semi-permeable hollow fibers) transgenic cell line secreting the mutant form of GLP-1 was shown to be an efficient system to deliver the hormone, and may be a promising therapy for type II diabetes (Burcelin et al., 1999).

Pancreatic beta cells death is induced by apoptosis. Transfer of the anti-apoptotic gene bcl-2 into beta cells prevented their apoptosis and was shown to have the potential to prevent autoimmune beta cell destruction and destruction of transplanted beta cells (Liu et al., 1996).

Pancreatic beta cells are characterized by poor growth capacity. Therefore, when beta cells are eliminated in diabetes no regeneration is detected. Although pancreatic stem cells were shown to reside in the pancreatic ductual epithelium (Bonner-Weir et al., 2000), islet neogenesis in diabetes animal was not observed. However, stimulation of beta cell neogenesis and replication of existing beta cells might be an effective strategy in diabetes treatment. Since Pdx1-positive progenitor cells were shown to differentiate into insulin-producing cells after islet elimination (Fernandes et al., 1997), activation or insertion of Pdx1 into progenitor cells could be a possible approach to increase the number of beta cells. Other genes that may

be considered include members of the *reg* gene family that were also shown to be involved in regeneration processes in animal models (Bone et al., 1997).

5.3. Transplantation of insulin-producing cells

5.3.1. Animal models of diabetes

To test, whether *in vitro* generated cells represent functional cell type that may potentially be clinically applicable, animal disease models with pathologic mechanisms sufficiently similar to those of human diseases were developed. The animal diseases may be either induced or naturally occurring. The non-obese diabetic (NOD) mouse represents an animal model of spontaneously developing type I diabetes (Atkinson and Leiter, 1999). In NOD mice insulin-producing beta cells are destroyed during an autoimmune process similar to the process occurring in humans. Destruction of pancreatic beta cells may also be induced in animals by multiple low dose injections of streptozotocin (STZ) (Like and Rossini, 1976). The dose of STZ depends on the animal strain used. The STZ model allows the use of appropriate mouse strains to minimize the risk of immune rejections after transplantation of cells into animals with different genetic backgrounds. The disadvantage of use of STZ is its toxicity to other organs, such as kidney and liver.

5.3.2. Transplantation of islets and whole pancreas

One of the most promising therapeutic option for the treatment of diabetes is the transplantation of insulin-producing cells. Several different cell sources are considered as successful replacement therapies. Transplantation of the whole pancreas or pancreatic islets can result in insulin independence, but problems related to graft rejections are reasons for a very low efficiency of this therapy (Brendel et al., 1999). Islet allografts usually mismatch for at least one MHC antigen, therefore without any immunosuppression, these allografts are

rejected. Liver transplantation is known to improve immunological tolerance, therefore simultaneous liver and islet transplantations are considered for some patients (especially with liver failure) and was shown to increase islet allograft survival (Titus et al., 2000). A specific glucocorticoid-free immunosuppression combined with engraftment of an adequate islet mass (Edmonton protocol) allowed to overcome these problems and rescued diabetes without symptoms of graft rejections and without need for exogenous insulin injections (Shapiro et al., 2000). However, for successful transplantation freshly isolated islets from at least two immunologically compatible donors are necessary. Because the number of human donors is limited, wide scale application of this therapy is not possible. The pool of donor organs needs to be extended. This could include also the use of tissues from other species. Pigs represent the most suitable donor species. The advantage of porcine tissue would be unlimited source of islets and the possibility to perform genetic manipulations, but requirement of strong immunosuppression and the risk of retroviral infections create barriers for clinical applications (Butler, 1998). Therefore, other cell sources must be considered to generate transplantable insulin-producing cells for a therapy of diabetic patients.

5.3.3. Insulin-producing cell lines

The use of cells other than beta cells was further considered as a potential source of cells, especially in the contex that might not be recognized by the autoimmune response in type I diabetes (Lipes et al., 1997). Permanent insulin-expressing cell lines such as beta cell lines derived from insulinomas could represent an unlimited, easily available source of insulin-producing cells (Miyazaki et al., 1990). Transplantation of these cells in humans would require previous microencapsulation, however the injection of such immortalized cells may cause cancer in case of damage of the microencapsulation device. Many different cell types including fibroblasts, muscle cells, keratinocytes and hepatocytes were transfected with the insulin gene, however most of the engineered non-beta cells produced and secreted only

low levels of insulin and the glucose-controlled insulin release was not properly controlled. To improve insulin secretion in such systems, insulin expression driven by a promoter that responds to glucose was created (Mitanchez et al., 1997). Because insulin secretion in beta cells is an extremely rapid response to increased glucose levels, even a rapid transcriptional response from existing mRNA resulted in continued, inappropriate insulin secretion and led to hyper- or hypoglycaemia periods. A more sophisticated approach to obtain glucose-responsive cells was transfection of insulin expressing AtT20 cells with Glut-2 and glucokinase. Unfortunately, the maximum insulin release was attained only at subphysiological glucose concentrations (Hughes et al., 1993). Another approach considers the use of a specific fraction of enteroendocrine cells from gut called K cells, that are characterized by production and secretion of glucose-dependent insulinotropic peptide (GIP) in a similar manner as insulin in pancreatic beta cells (Damholt et al., 1999). Genetically engineered mice expressing insulin linked with GIP in K cells were rescued from the development of diabetes (Cheung et al., 2000). This approach could be used to generate donor cells *in vitro*, or to target insulin expression to non-beta cells *in vivo*. These findings demonstrate, that cell engineered approaches in principle, have the potential to generate cells with beta cells properties.

5.3.4. Embryonic stem cells

A promising alternative for the generation of transplantable insulin-producing cells are stem cell- and progenitor-based approaches. Stem cells are characterized by self-renewal capacity and the ability to differentiate into various different cells types. The definition includes embryonic and adult stem cells. ES cells are derived from the inner cell mass of the blastocyst and posses self-renewal potential paralleled by the maintenance of the undifferentiated state *in vitro*, when cultured on feeder layer and/or in the presence of interleukin-6 family cytokines, such as leukemia inhibitory factor (LIF) (Smith, 2001).

Another important characteristic is that single ES cells can give rise to colonies of genetically identical cells that have the same properties as the original cells. Because of these properties, ES cells are suitable targets for gene transfer technologies. The undifferentiated phenotype of mouse ES cells has been defined by several parameters including the expression of specific cell surface antigens (SSEA-1), membrane-bound receptors (gp130), transcription factors Oct-4 and Nanog, and of enzyme activities such as alkaline phosphatase (ALP) and telomerase [for rev. see (Czyz et al., 2003)].

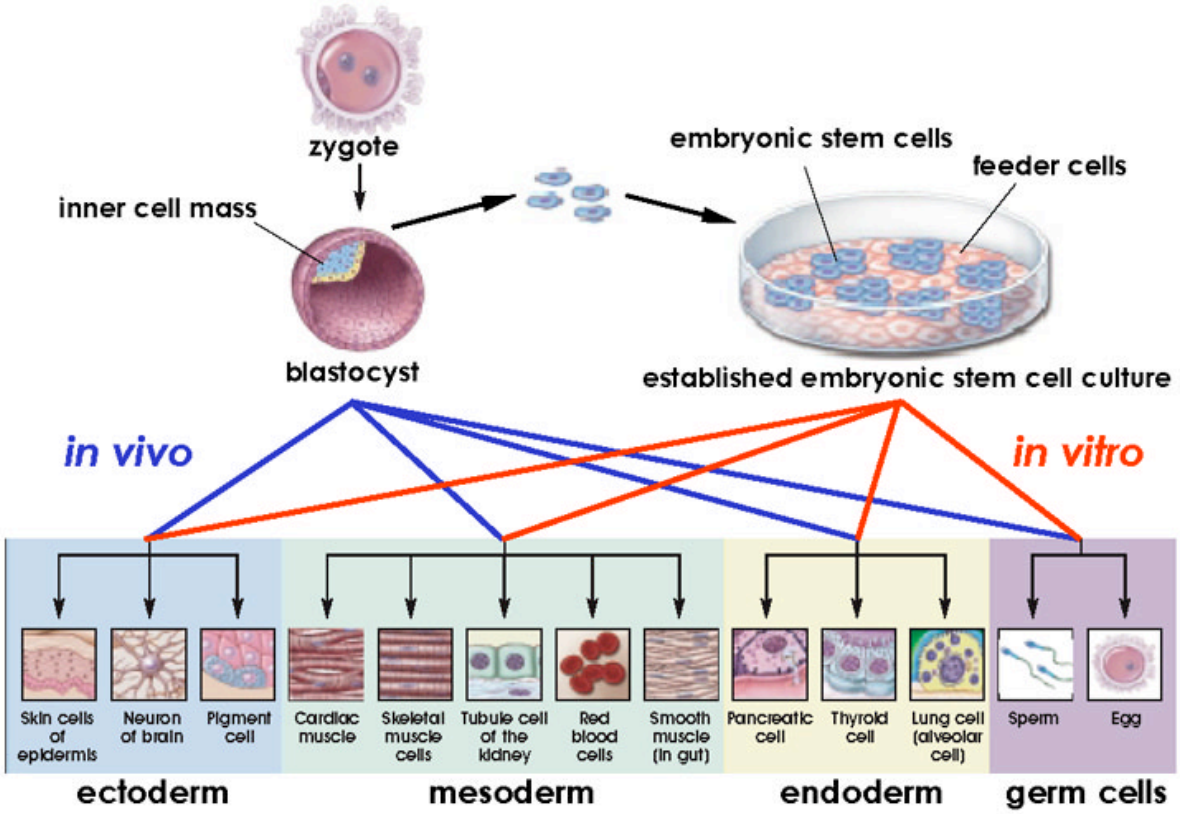

Fig. 5. Schematic representation of generation of embryonic stem cell cultures and comparison of developmental potential of blastocyst *in vivo* (blue lines) and embryonic stem cells *in vitro* [(red lines), a modified NIH report: "Stem cells: scientific progress and future research directions", 2001, http://www.nih.gov/]

ES cells have been proposed as having a tremendous potential as a powerful resource for cell replacement therapies, because of their pluripotency, and ability to differentiate into all cell types of the body (Fig. 5). When injected into blastocysts *in vivo*, ES cells contribute to all cell lineages of the developing embryo (Smith, 2001). *In vitro*, when cultivated as

embryo-like aggregates, called embryoid bodies (EBs), ES cells have the capability to differentiate into cellular derivatives of all three primary germ layers, i.e. endoderm, ectoderm and mesoderm. This aim is, however, dependent on the definition of culture conditions that direct the differentiation of ES cells into the desired lineage. Till now, several parameters have been defined to influence the developmental potency of ES cells in culture, such as: i) the number of cells differentiating in EBs ii) the composition of media and quality of sera, growth factors and other additives iii) cell density after differentiation induction and iv) ES cell lines used (Wobus et al., 2002). In appropriate conditions, ES cells differentiate into specialised and functional cells representing phenotypes of cardiogenic (Wobus et al., 1991; Wobus et al., 1997; Maltsev et al., 1993), myogenic (Miller-Hance et al., 1993; Rohwedel et al., 1994), vascular smooth muscle (Risau et al., 1988; Weitzer et al., 1995; Drab et al., 1997), neurogenic (Bain et al., 1995; Fraichard et al., 1995; Okabe et al., 1996), hepatic (Hamazaki et al., 2001; Jones et al., 2002; Yamada et al., 2002), haematopoietic (Wiles and Keller, 1991; Burkert et al., 1991; Keller et al., 1993; Hole, 1999), adipogenic (Dani, 1999), chondrogenic (Kramer et al., 2000), epithelial (Bagutti et al., 1996), and endothelial (Risau et al., 1988; Wang et al., 1992) lineages. Recently, the *in vitro* differentiation of ES cells into male and female gametes were reported (Hubner et al., 2003; Geijsen et al., 2004).

The first successful differentiation of mouse ES cells into the pancreatic lineage was attained by transfection of a drug resistance gene under the control of the insulin promoter, followed by cell lineage-selection and maturation. After *in vitro* differentiation, one transgenic ES cell clone showed regulated insulin release, and after transplantation, normalized glycaemia in streptozotocin-induced diabetic mice (Soria et al., 2000). Pancreatic differentiation from ES cells was also reported by application of a sophisticated five-stage method including the formation of embryoid bodies (EBs), selection and propagation of progenitors expressing the intermediate filament protein nestin and final maturation (Lumelsky et al., 2001). Modification of this protocol combined with the introduction of the

Pax4 gene into ES cells (Blyszczuk et al., 2003) or treatment of ES cells with an inhibitor of phosphoinositide 3-kinase (PI3K) during terminal differentiation (Hori et al., 2002) resulted in the generation of functional islet-like clusters [for detailed description and discussion of ES-derived pancreatic differentiation, see (Blyszczuk and Wobus, 2004)].

However, a feature of undifferentiated ES cells is their tumouroigenic potential. When ES cells are transplanted into syngeneic animals, they form teratomas or teratocarcinomas (Stevens, 1983; Wobus et al., 1984). We showed that the oncogenic properties of ES cells are not eliminated during pancreatic differentiation *in vitro* (Blyszczuk et al., 2003). However, it is unclear, whether tumours are formed from the remaining undifferentiated fraction of ES cells, or wether ES-derived pancreatic cells still posses a tumourigenic potential. The tumour formation capacity of ES-derived cells is the main obstacle in the development of ES-based cell therapies.

5.3.5. Adult stem and progenitor cells

A potential alternative to ES cells are adult stem (AS) cells located in adult tissues where they are involved in the maintenance and regeneration of tissues. AS cells are characterized by a developmental flexibility (Theise and Wilmut, 2003) and were found not only in tissues characterized by extensive regeneration potential, such as the haematopoietic system (de Haan, 2002), skin (Watt, 2001), liver (Theise et al., 1999) or intestine (Potten, 1998), but also in tissues traditionally considered as having no regenerative potential. These tissues include for example, the central nervous system (Okano, 2002), retina (Tropepe et al., 2000), skeletal muscle (Seale et al., 2001) and pancreas (Bonner-Weir and Sharma, 2002). Numerous reports of the last years postulated that AS cells do not contribute only to cell types of their own tissue, but were shown to trans-differentiate into various cell types of other lineages. However it was always found that trans-differentiation capacity was rather limited [for rev. (Czyz et al., 2003)]. Moreover, experiments of the last years presented evidences that

many of the transdifferentiation properties were the consequences of cell fusions between donor and recipient cells (Alvarez-Dolado et al., 2003; Vassilopoulos et al., 2003; Wang et al., 2003).

The advantage of use of AS cells for tissue regeneration would be their low tumourigenic potential and the autologous source of the graft. The propagation and direction of multipotent AS cells into the pancreatic lineage *in vitro* would allow to generate a sufficient amount of transplantable cells. A successful generation of pancreatic cells was reported from different sources including pancreatic ductal epithelium (Ramiya et al., 2000; Bonner-Weir et al., 2000; Yao et al., 2004), nestin-positive cells derived from islets (Zulewski et al., 2001; Abraham et al., 2002), oval cells (Yang et al., 2002), intestine epithelium (Suzuki et al., 2003) and bone marrow (Ianus et al., 2003; Oh et al., 2004) [for a detailed description and discussion of AS-derived pancreatic differentiation see (Blyszczuk and Wobus, 2004)].

Use of stem cells derived from easily accessible tissues such as bone marrow seems to be an optimal strategy for the generation of autologous cells to repair tissue damages. Until now, the major problem in the use of AS cells are their limited proliferation as well as inefficient and limited differentiation capacities.

The normalization of blood glucose levels in cell replacement strategies is obviously the goal of all these procedures. However, until now there has been very little attention paid to other aspects of islets function, including the regulatory role of glucagon, somatostatin and pancreatic polypeptides in carbohydrate, protein and lipid metabolism and certainly we are far away from any therapeutic application of stem cells in diabetes therapy.

6. Role of nestin in pancreatic differentiation in vivo and in vitro

The identification of pancreatic progenitor cells is a key aspect of stem cell research related to the cell therapy in diabetes. Due to limited transdifferentiation capacity of AS cells, the characterization of multipotent stem and progenitor cells in different tissues is desirable.

Nestin-expressing cells are considered as a potential population of such multipotent progenitor cells. In pancreatic islets, only a small subset of cells express nestin and these cells were proposed to represent precursors of differentiated pancreatic endocrine cells (Hunziker and Stein, 2000). However, in islets nestin-expressing cells were negative for insulin and during pancreas development nestin was not detected in epithelial cells. Instead, nestin expression was found to be restricted to mesenchymal cells closely associated with islets. These nestin-positive cells residing in islets are called pancreatic stellate cells and were proposed to play an important role in the growth and maintenance of islets (Lardon et al., 2002; Selander and Edlund, 2002; Klein et al., 2003; Treutelaar et al., 2003). Recently a small population of cytokeratin 19-positive cells was reported to co-express nestin in human pancreatic ductal cells (Street et al., 2004).

The involvement of nestin in the pancreatic development was carefully studied by lineage-tracing experiments. Nestin was found to be transiently expressed in undifferentiated pancreatic epithelial progenitors that contributed mainly to the formation of exocrine acinar cells (Delacour et al., 2004; Esni et al., 2004).

In the lineage-tracing experiments a promoter/enhancer regulatory element containing the second intron of the *nestin* gene was used. This transcription regulator located in the second intron was reported to regulate nestin expression in neural precursors, whereas in muscle precursor cells this transcription regulator was shown to be much less active (Zimmerman et al., 1994). Considering that the *nestin* gene is complex (Yang et al., 2001) and that cell type-specific regulatory elements of *nestin* exist, one can not exclude an alternative transcriptional regulation during the development of endocrine pancreas versus neuronal or muscle tissues. Therefore, until now, the involvement of nestin in endocrine pancreatic development has not been definitively elucidated.

Remarkably, when nestin-positive cells derived from pancreatic islets were cultured *in vitro*, they showed extended proliferation capacity and the ability to differentiate into cells

expressing pancreatic endocrine, exocrine and hepatic markers (Zulewski et al., 2001; Abraham et al., 2002). Expression of nestin after *in vitro* culture was found in other cells isolated from adult organism including bone marrow (Kabos et al., 2002), cord blood (Buzanska et al., 2002) or corneal limbal epithelium (Zhao et al., 2002). Furthermore, the *in vitro* generation of pancreatic islet-like clusters from ES cells include the selection of nestin-positive progenitors (Lumelsky et al., 2001; Blyszczuk et al., 2003; Hori et al., 2002). Selection of nestin-expressing progenitors was also successfully used for the induction of ES cells into neuronal (Okabe et al., 1996) and hepatic phenotypes (Kania et al., 2003).

Nestin is an intermediate filament protein, which was initially identified as a marker of neural stem and progenitor cells (Lendahl et al., 1990). However, nestin is transiently expressed not only during development of the central nervous system (Frederiksen and McKay, 1988; Lendahl et al., 1990; Hockfield and McKay, 1985), but also in different cell types of embryonic and adult tissues, such as developing skeletal muscle (Sejersen and Lendahl, 1993), mesenchymal cells (Selander and Edlund, 2002), cardiomyocytes (Kachinsky et al., 1995) or endothelial cells (Mokry and Nemecek, 1998a). Nestin seems to play also an important role during regeneration processes. In response to injury, the expression of nestin is induced and enhanced in various tissues including central nervous system (Clarke et al., 1994; Kim et al., 2003), liver (Niki et al., 1999), pancreas (Lardon et al., 2002) or skeletal muscle (Vaittinen et al., 2001) systems. However, molecular mechanisms of the activation of nestin expression after injury remains unknown.

The functional role of nestin has not been determined yet. Intermediate filament proteins form dynamic structures that change their intracellular organization during various conditions, such as mitosis, differentiation and different pathological situations. It was suggested that nestin plays a role in various proliferation and migration processes in progenitor cells (Mokry and Nemecek, 1998b). In neuroepithelial cells, nestin was shown to be responsible for the organization and the maintenance of the elongated cell shape (Matsuda

et al., 1996). It was also suggested that the expression of nestin may be associated with an increase in cytoplasmic trafficking required for progenitor cells undergoing rapid rounds of division (Chou et al., 2003).

Transient expression of nestin in progenitor cells during development of various tissues, expression induction after *in vitro* culture or upon injury and involvement in ES cell-derived differentiation emphasize the potential of nestin-expressing cells as a multipotent progenitor cell type. Particularly, nestin-positive cells seem to be a suitable source for the generation of insulin-producing cells and might serve as a potential cellular source for future cell replacement therapies in diabetes.

7. Summary of results

The aim of this study was to establish methods and strategies to differentiate mouse ES cells into insulin-producing beta-like cells and to characterize intermediate and terminal ES cell progeny. ES cells of wild type and ES cells constitutively expressing Pdx1 or Pax4 were comparatively studied. Four different culture systems were developed to investigate pancreatic differentiation *in vitro*: i) spontaneous differentiation of ES cells without induction of pancreatic differentiation [1], ii) selection of nestin-positive progenitors followed by induction of pancreatic differentiation [1,2,5], iii) spontaneous differentiation of ES cells into multi-lineage progenitors followed by induction of pancreatic differentiation [3,4] and iv) histotypic formation of spheroids from islet-like clusters [1,5; numbers in brackets refer to the original papers].

7.1. Influence of constitutive expression of Pax4 and Pdx1 on spontaneous differentiation of ES cells

Wild type (wt) ES cells and ES cells constitutively expressing Pdx1 and Pax4 were differentiated *in vitro* and comparatively studied for the presence of cardiac, skeletal muscle, and neuronal cells, as well as by RT-PCR and immunofluorescence for pancreas-specific genes expression. Pax4+ cells showed delayed and reduced cardiac differentiation and a lower degree of skeletal and neuronal differentiation in comparison to wt cells, whereas constitutive expression of Pdx1 only modestly affected the ES cell differentiation pattern [1].

Important changes in the expression levels of pancreatic genes were detected in Pax4+, and to a lesser extent, Pdx1+ cells. Semi-quantitative RT-PCR analyses revealed an up-regulation of ngn3, Pax6 and insulin mRNA levels in Pdx-1+ cells, whereas isl-1, ngn3, insulin, IAPP and Glut-2, but not Pdx1, mRNA levels, were up-regulated in Pax4+ cells. Pax6 mRNA levels were increased in Pax4+ cells only at intermediate stages, but down-regulated at terminal stages. Pax4 transcripts were absent in wt and Pdx1+ cells [1].

Immunofluorescence analysis of insulin, glucagon, somatostatin and pancreatic polypeptide showed that wt ES cells spontaneously differentiated into cells predominantly co-expressing all four pancreatic hormones, whereas few cells were positive for insulin only. No significant changes were detected in the proportion of insulin- versus glucagon-positive cells during all differentiation stages. Pax4+ cells revealed an up to 4.5-fold, and Pdx1+ cells an up to 2-fold, increase in the level of insulin-positive cells, whereas the level of glucagon-positive cells was not altered in both transgenic cell lines in comparison to wt cells [1].

These results showed that Pax4 and to lesser extent Pdx1, affect the spontaneous ES cell differentiation promoting development into the pancreatic lineage.

7.2. Generation of islet-like clusters from ES-derived nestin-positive progenitors

Immunofluorescence studies on spontaneously differentiating ES cells showed that constitutive expression of Pax4, but not Pdx1 significantly increased the number of nestin-positive cells at intermediate stages in comparison to wt cells. Therefore, in subsequent experiments, wt and Pax4+ ES cells were cultured under conditions selective for the development of nestin-positive cells followed by induction of pancreatic differentiation [5]. After the selection step, Pax4+ cells generated a significantly higher number of nestin-positive cells. Continued differentiation resulted in the formation of insulin-positive islet-like clusters at terminal stages [1]. Double immunofluorescence studies showed that most of the insulin-positive cells co-expressed C-peptide confirming that ES-derived cells were capable to differentiate into pancreatic cells producing *de novo* insulin. However, a small fraction (10 – 15%) of ES-derived insulin-positive cells was negative for C-peptide, but showed fragmented nuclei suggesting apoptosis [2]. These cells might represent the population that concentrates insulin from the medium, but not produces insulin [see (Rajagopal et al., 2003)]. Detection of Pax4, insulin, Glut-2 and IAPP mRNA transcripts by RT-PCR in wt ES-derived cells at terminal stages confirmed pancreatic differentiation [2,5].

Pax4+ cells generated a significantly higher amount of insulin-positive cells (up to ~80% of total cells) in comparison to wt cells (up to ~20-25%) and the measurement of intracellular insulin levels by ELISA showed a significant increase in Pax4+ cells (98.7 ng insulin/mg protein), compared to wt cells (20.7 ng insulin/mg protein) at terminal stage of differentiation. Both wt and Pax4+ cells secreted insulin in response to glucose or tolbutamide (a sulfonylurea known to stimulate insulin secretion), but no significant differences of the secreted/intracellular insulin level between both cell lines were found [1].

The functional potential of ES-derived insulin-producing cells was investigated in transplantation experiments. Wt and Pax4+ cells at terminal stage of differentiation were transplanted under the kidney capsule and into the spleen of STZ-induced diabetic animals. Mice transplanted with wt or Pax4+ ES cells retained normal blood glucose levels over a period of 14 days, whereas non-transplanted control animals became hyperglycaemic 48 hrs following STZ treatment. 14 days after transplantation, numerous insulin-positive cells were observed in the kidney capsule and spleen of animals transplanted with wt and Pax4+ cells [1]. These data showed that functional islet-like clusters can be generated from ES cells via nestin-positive progenitors and the process is promoted by constitutive expression of Pax4.

7.3. Histotypic generation of spheroids from differentiated ES cells

The three-dimensional histotypic culture system was adapted for the differentiation and maturation of wt and Pax4+ ES-derived pancreatic cells generated from the nestin-positive progenitors. Cultivation in “spinner” culture flasks resulted in cell aggregation and spheroid formation. In immunofluorescence and ELISA studies, the accumulation of insulin and glucagon in spheroids was detected, whereas insulin release was reduced to undetectable levels. Immune electronmicroscopy studies of Pax4+ spheroids showed insulin-positive secretory granules comparable to the granules of embryonic beta cells. In wt spheroids no secretory granules were found and only occasionally cytoplasmic insulin labelling [1]. The

lack or low levels of insulin release of spheroid-derived cells let us to conclude that the histotypic culture system alone is not sufficient for the generation of functional ES-derived pancreatic beta-like cells.

7.4. Generation of islet-like clusters without selection of nestin-positive progenitor cells

The differentiation protocol including selection of nestin-positive progenitors was shown to be irreproducible (Rajagopal et al., 2003). Therefore we established a new differentiation protocol, where the selection of nestin-positive progenitors was replaced by the spontaneous generation of early multi-lineage progenitor cells followed by the differentiation induction into the pancreatic lineage. This protocol allowed the investigation of progenitors involved in pancreatic differentiation *in vitro*. Upon induction of pancreatic differentiation, a significant up-regulation of Pdx1, Pax4, insulin and IAPP mRNA levels were found, demonstrating the induction of pancreatic differentiation in ES cell cultures. Similarly as shown with the protocol for selection of nestin-positive progenitors, this method also resulted in the generation of islet-like clusters co-expressing insulin and C-peptide in most of the ES-derived cells [3].

The functional properties of ES-derived wt and Pax4+ cells were analysed by electrophysiological analysis and by ELISA. Electrophysiological measurements using the patch-clamp technique showed that ES-derived pancreatic cells were excitable and revealed similar properties as embryonic beta cells with respect to voltage-activated Na^+ channel inactivation, voltage-activated K^+ channel and the Ca^{2+}-dependent secretory activity. In some Pax4+ cells, K_{ATP} channels were responsive to the specific channel opener diazoxide suggesting the development of beta-like cells. Measurement of insulin content by ELISA showed that both, wt and Pax4+ cells secreted insulin in response to high glucose concentrations. Pax4+ cells showed a higher intracellular insulin level, but the ratio of released/intracellular insulin level was similar for wt and Pax4+ cells. The higher insulin

levels of Pax4+ cells in comparison to wt cells correlated with the higher levels of C-peptide expression in Pax4+ cells [3].

In transplantation experiments, the *in vivo* potential of ES-derived insulin-producing wt and Pax4+ cells to normalize hyperglycemia in STZ-induced diabetic mice was tested. Cells were transplanted under the kidney capsule and into the spleen of hyperglycemic mice with blood glucose levels above 15 mM over a period of about 5 weeks. Mice transplanted with wt cells and sham-transplanted control animals remained hyperglycemic at levels of about 25- 30 mM and suffered from diabetic weight loss. Animals transplanted with Pax4+ cells reduced blood glucose levels 2 weeks after transplantation and attained a normal glycaemia status by 5 weeks. Weight loss was attenuated in mice engrafted with Pax4+ cells. Partial graft removal by unilateral nephrectomy performed 26 days after transplantation resulted in increased blood glucose level in contrast to non-nephrectomized mice [3]. These data obtained with the new protocol show that selection for nestin positive cells is neither required nor profitable for the generation of islet-like clusters from ES-cells.

7.5. Characterization of progenitor cells involved in ES-derived pancreatic differentiation

To characterize progenitors involved in pancreatic differentiation of ES cells *in vitro*, we used the protocol which generates multi-lineage progenitors followed by differentiation induction into the pancreatic lineage. Differentiating wt and Pax4+ cells were analysed before induction of pancreatic differentiation (= multi-lineage progenitor stage) and after induction of pancreatic differentiation (= committed pancreatic progenitor stage) and continued differentiation into islet-like clusters (= terminal stage).

Double immunofluorescence analysis showed that nestin and C-peptide were highly abundant and were co-expressed at the committed pancreatic progenitor stage, whereas C-peptide-positive islet-like clusters were negative for nestin at the terminal stage. It indicated

that nestin was involved and transiently expressed during pancreatic differentiation *in vitro* [3].

Immunofluorescence studies using nestin- and desmin-specific antibodies revealed a partial co-expression of these proteins at the multi-lineage progenitor stage. Upon induction of pancreatic differentiation, the amount of double nestin- and desmin-positive cells was significantly reduced, suggesting that desmin is not involved in the terminal differentiation of pancreatic cells [3].

RT-PCR analysis revealed constant cytokeratin (CK) 19 mRNA levels at all analysed stages of differentiation. After induction of pancreatic differentiation, CK19 failed to form filament, but resembled dot-like structures. Independent double immunofluorescence analyses showed that CK19 is highly co-expressed with C-peptide and nestin at the committed pancreatic progenitor stage. At the terminal stage, some C-peptide-positive cells within islet-like clusters were negative for CK19 that may indicate partial maturation of ES-derived cells during differentiation [3].

Immunofluorescence analysis showed that Isl-1 is only transiently expressed in the nestin-positive subpopulation of committed pancreatic cells [3].

Carbonic anhydrase II, a protein expressed in pancreatic ducts, revealed high co-expression with C-peptide in the committed pancreatic progenitors and at terminal stage. These results suggest that *in vitro* generated ES-derived cells represent an immature pancreatic cell population, because carbonic anhydrase II is not expressed in the definitive mature pancreatic beta cells [3].

In conclusion, the data show that genes involved in pancreatic beta cell development and function *in vivo,* such as *CK19, Isl-1, carbonic anhydrase II, Pax4, IAPP, Pdx1 and insulin* are also expressed during ES-derived pancreatic differentiation *in vitro*. Furthermore, nestin was shown to be transiently involved in the generation of islet-like clusters from ES cells.

8. Conclusions

Our studies showed that ES cells are able to differentiate *in vitro* into islet-like clusters characterized by the expression of tissue-specific genes, ion channel activity similar to embryonic beta cells, glucose-dependent insulin release and the ability to rescue diabetes in an animal model.

Pax4 was shown to promote ES-derived pancreatic differentiation *in vitro.* Our results suggest that Pax4 may play a significant role in directing undifferentiated ES cells into the pancreatic lineage and should be considered in pancreatic differentiation systems employing adult stem cells.

The transient expression of nestin during pancreatic differentiation *in vitro* implies that nestin is involved in ES-derived pancreatic differentiation, which suggests a different expression of nestin in *in vivo* and in *in vitro* systems.

This study showed that the generation of ES-derived pancreatic insulin-producing cells is possible. This opens new strategies for a large-scale production of insulin-producing cells, one of the requirements of cell-replacement therapies for the treatment of diabetes. However, before clinincal applications become possible, problems concerning terminal maturation and the purity of the transplanted cells have still to be solved. To this aim, exogenous factors, components of the ‘niche’ of islets have to be applied to promote maturation of ES-derived progeny, and the application of selection strategies to generate pure populations of insulin-producing cells is necessary.

9. List of publications and manuscripts on which thesis is based and declaration on the contributions

[1] - Blyszczuk P, Czyz J, Kania G, Wagner M, Roll U, St-Onge L, Wobus AM. (2003) Expression of Pax4 in embryonic stem cells promotes differentiation of nestin-positive progenitor and insulin-producing cells. *Proc. Natl. Acad. Sci. USA*, 100, 998-1003

Most of experimental work was done by myself. Pax4 and Pdx1 constructs were generated by Dr. U. Roll (Develogen AG, Göttingen), electron microscopy was performed by Dr. M. Wagner (University of Ulm), transplantation was done together with Dr. L. St-Onge (Develogen AG, Göttingen) and the histotypic spheroid culture was developed together with Dr. G. Kania.

[2] - Blyszczuk P and Wobus AM. Stem cells and pancreatic differentiation *in vitro*. *J. Biotechnology* (in press)

Experimental work was done by myself. Review part was written by myself, in collaboration with Prof. Dr. A Wobus.

[3] - Blyszczuk P, Asbrand C, Rozzo A, Kania G, St-Onge L, Rupnik M and Wobus AM. Generation of functional beta-like cells from embryonic stem cells and characterization of pancreatic progenitor cells expressing nestin Int. J. Dev. Biol. (in press)

Most of experimental work was done by myself. Electrophysiological studies were performed by Dr. A. Rozzo and Dr. M. Rupnik (both European Neuroscience Institute, Göttingen) and transplantation was done together with Dr. C. Asbrand (Develogen AG, Göttingen).

[4] - Blyszczuk P, Kania G and Wobus AM. Pancreatic and hepatic ES cell differentiation *in vitro*. Chapter 9 in *Embryonic stem cells – a practical approach.* Eds. E. Notarianni and M. Evans, *Oxford University Press*. (in press)

Description of ES cell culture and pancreatic differentiation was done by myself and hepatic differentiation was done by Dr. G. Kania, all parts in collaboration with Prof. Dr. A. Wobus.

[5] - Kania G, Blyszczuk P, Czyz J, Navarrete-Santos A, Wobus AM. (2003) Differentiation of mouse embryonic stem cells into pancreatic and hepatic cells. *Methods in Enzymology* 365, 287-303

Experimental and theoretical parts of pancreatic differentiation were done by myself.

[6] - Rolletschek A, Blyszczuk P and Wobus AM. (2004) Embryonic stem cell-derived cardiac, neuronal and pancreatic cells as model systems to study toxicological effects. *Toxicology Letters,* 149 (1-3), 361-369

Parts describing pancreatic differentiation were written by myself.

10. Zusammenfassung der wichtigsten Ergebnisse und Schlußfolgerungen

Ziel der Arbeit war die Etablierung von Methoden und Strategien zur Differenzierung von embryonalen Stamm(ES)-Zellen der Maus in Insulin-produzierende pankreatische Zellen, sowie die Charakterisierung verschiedener Entwicklungsstadien. ES-Zellen, die die Pankreas-Entwicklungskontrollgene Pdx1 bzw. Pax4 konstitutiv exprimieren (Pdx1+, Pax4+) wurden vergleichend zu Wildtyp (Wt)-ES-Zellen analysiert. Vier verschiedene Kultivierungssysteme wurden entwickelt, um die Pankreas-Differenzierung *in vitro* zu untersuchen: i) spontane Differenzierung von ES-Zellen ohne Induktion der pankreatischen Differenzierung [1], ii) Selektion von Nestin-positiven Vorläuferzellen gefolgt von der Induktion der pankreatischen Differenzierung [1,2,5], iii) spontane Differenzierung von ES-Zellen in Vorläuferzellen aller drei Keimblätter (multi-lineage progenitors) gefolgt von der Induktion der pankreatischen Differenzierung [3,4] und iv) die histotypische Entwicklung von Sphäroiden aus Insel-ähnlichen Clustern [1,5; Zahlen in Klammern verweisen auf die Original-Arbeiten].

Einfluss der konstitutiven Expression von Pax4 und Pdx1 auf die spontane Differenzierung von ES Zellen in pankreatische Zellen

Wt, Pdx1+ und Pax4+ ES Zellen wurden nach spontaner Differenzierung mittels morphologischer Analyse auf die Anwesenheit von Herz-, Skelettmuskel- und Nervenzellen untersucht. Parallel dazu wurden pankreasspezifische Genexpressionsmuster mit Hilfe von RT-PCR- und die Bildung pankreasspezifischer Proteine mit Immunfluoreszenz-Analyse untersucht. Pax4+ Zellen zeigten im Vergleich zu Wt-Zellen verzögerte und verringerte Herzzelldifferenzierung und eine verminderte Differenzierung in Skelettmuskel- und neuronale Zellen, während die konstitutive Expression von Pdx1 nur geringfügig das ES-Zelldifferenzierungsmuster veränderte (1). Wichtige Veränderungen in der Expressionsintensität von pankreasspezifischen Genen wurden in Pax4+ und in geringerem

Maß in Pdx1+ Zellen beobachtet. Semi-quantitative RT-PCR-Analyse demonstrierte eine Erhöhung der Transkriptmenge der pankreasspezifischen Gene ngn3, Pax6 und Insulin in Pdx1+ Zellen, wohingegen isl-1, ngn3, Insulin, IAPP und Glut-2 (nicht jedoch Pdx1) mRNA in Pax4+ Zellen hochreguliert wurden. Pax4-Transkripte waren in Wt- und Pdx1+ Zellen nicht nachweisbar [1]. Immunfluoreszenz-Analyse zeigte, dass die pankreasspezifischen Hormone Insulin, Glucagon, Somatostatin und pankreatisches Peptid (PP) in differenzierten Wt-Zellen ko-exprimiert waren, und nur wenige Zellen ausschließlich Insulin bildeten. Pax4+ Zellen zeigten dagegen eine 4.5- fache und Pdx1+ Zellen eine zweifache Erhöhung der Menge Insulin-positiver Zellen, während der Anteil von Glucagon-positiven Zellen in beiden Linien im Vergleich zu Wildtypzellen unverändert war [1]. Diese Ergebnisse zeigten, dass Pax4 (und in geringerem Maß, Pdx1) die spontane Differenzierung von ES-Zellen in die pankreatische Linie positive beeinflussen.

Bildung von Insel-ähnlichen Clustern aus ES-Zell-abgeleiteten Nestin positiven Vorläuferzellen

Immunfluoreszenz-Analyse an spontan differenzierenden ES-Zellen zeigte, dass die konstitutive Expression von Pax4 die Anzahl von Nestin-positiven Zellen im Vergleich zu Wt-Zellen erhöhte. Deshalb wurden in aufeinanderfolgenden Experimenten Wt- und Pax4+ ES-Zellen unter Selektionsbedingungen kultiviert, die die Entwicklung von Nestin-positiven Zellen fördern. Anschließend wurde in diesen Zellen pankreatische Differenzierung induziert. Nach dieser Selektion wurden in Pax4+ Zellen eine signifikant höhere Anzahl von Nestin-positiven Zellen - und nach anschließender pankreatischer Differenzierung - eine erhöhte Anzahl von Insulin-positiven Inselzell-ähnlichen Clustern gebildet [1].

Immunfluoreszenz-Studien zeigten, dass die meisten der Insulin-positiven Zellen C-Peptid ko-exprimierte, womit nachgewiesen wurde, dass ES-Zellen in pankreatische Zellen differenzieren, die *de novo* Insulin produzieren. Lediglich ein Anteil von 10-15% der

differenzierten Insulin-positiven Zellen war C-Peptid-negativ. Diese Zellen wiesen fragmentierte Kerne auf, was auf Apoptose-Prozesse schließen lässt [2]. Bei diesen Zellen handelt es sich offensichtlich um Zellen, die Insulin anreichern, aber nicht selbst produzieren (siehe Rajagopal et al., 2003). Der Nachweis von Pax4, Insulin, Glut-2 und IAPP mRNA durch RT-PCR in differenzierten Wt ES-Zellen bestätigte eine erfolgreiche Differenzierung in pankreatische Zellen [2,5].

Pax4+ Zellen bildeten eine signifikant höhere Anzahl von Insulin-positiven Zellen (bis zu ~80% aller Zellen) im Vergleich zu Wt-Zellen (bis zu 20-25% aller Zellen). Die Messung der intrazellulären Insulinmenge mittels ELISA zeigte einen signifikanten Anstieg in Pax4+ Zellen im Vergleich zu differenzierten Wt-Zellen. Sowohl Wt- als auch Pax4+ Zellen gaben Insulin nach Einwirkung hoher Konzentrationen von Glukose ab. Dagegen wurden keine signifikanten Unterschiede in der Rate von sezerniertem und interzellulärem Insulin zwischen beiden Linien gefunden [1].

Das funktionelle Potential der Insulin-produzierenden Zellen wurde nach Transplantation in ein diabetisches Mausmodell untersucht. Differenzierte Wt- und Pax4+ Zellen wurden in Niere und Milz von Streptozotocin (STZ)-behandelten diabetischen Tieren transplantiert. Die mit Wt- oder Pax4+ ES-Zellen transplantierten Mäuse zeigten innerhalb von 14 Tagen eine Normalisierung des Blutzuckerspiegels, während nicht transplantierte Kontrolltiere 48 Stunden nach der STZ-Behandlung hyperglämisch wurden. 14 Tage nach der Transplantation wurden zahlreiche Insulin-produzierende Zellen unter der Nieren-Kapsel und in der Milz transplantierter Tieren nachgewiesen. [1]. Diese Daten zeigen, dass aus ES-Zellen entwickelte pankreatische Zellen funktionell sind und Insel-ähnliche Cluster über Nestin-positive Vorläuferzellen erzeugt werden können. Der Entwicklungsprozess wird durch konstitutive Expression von Pax4 gefördert.

Histotypische Bildung von Sphäroiden aus differenzierten ES Zellen

Es wurde ein histotypisches Differenzierungsmodell zur Entwicklung von komplexen dreidimensionalen Aggregaten (Sphäroiden) aus Wt- und Pax4+ Zellen in Spinner-Kultur entwickelt. Durch Immunfluoreszenz-Analyse und nach ELISA wurden eine hohe Synthese und Speicherung (jedoch nur eine geringe Abgabe) von Insulin und Glukagon aus den Sphäroiden nachgewiesen. Die immunelektronenmikroskopische Untersuchung der Sphäroide zeigte in Pax4+ (nicht jedoch in Wt-) Zellen Insulin-positive sekretorische Granula, die denen von embryonalen Beta-Zellen vergleichbar waren. Daraus kann geschlussfolgert werden, dass die histotypische Differenzierung für die Entwicklung von funktionellen pankreatischen Beta-Zellen allein nicht ausreichend ist.

Die Erzeugung Insel-ähnlicher Cluster ohne Selektion Nestin-positiver Vorläuferzellen

In vielen Untersuchungen (siehe Rajagopal et al., 2003) war das Differenzierungprotokoll, das auf der Selektion Nestin-positiver Vorläuferzellen beruht, nicht reproduzierbar. Aus diesem Grund etablierten wir ein neues Differenzierungprotokoll, bei dem die Selektion Nestin-positiver Zellen durch die Entwicklung von Vorläuferzellen aller drei Keimblätter ersetzt wurde. Diese Vorläuferzellen wurden danach in die pankreatische Linie differenziert. Das Protokoll ermöglichte die Untersuchung der Rolle der Vorläuferzellen während der pankreatischen Differenzierung *in vitro*. Nach Aktivierung der pankreatischen Differenzierung wurde eine signifikante Hochregulierung der Trankriptmengen von Pdx1, Pax4, Insulin und IAPP gefunden und damit die pankreatiche Differenzierung in ES-Zellen nachgewiesen. Auch mit dieser Methode wurden Insel-ähnliche Cluster entwickelt, die Insulin und C-Peptid in den meisten Zellen aufwiesen, während nur ein geringer Anteil Insulin-positiver Zellen kein C-Peptid bildete.

Die funktionellen Eigenschaften von Wt- und Pax4+ Zellen wurden mit elektrophysiologischen Untersuchungen mit Hilfe der „patch-clamp“ Technik und mit ELISA analysiert. Die elektrophysiologischen Messungen zeigten, dass aus ES-Zellen abgeleitete

pankreatische Zellen die gleichen Eigenschaften wie embryonale Beta-Zellen aufwiesen, nämlich spannungsaktivierte Na^+ und K^+ Kanäle und Ca^{2+}-abhängige sekretorische Aktivität. In einigen Pax4+ Zellen wurden ATP-modulierte K-Kanäle gemessen, was auf einen höheren Reifungsgrad der Pax4+ Zellen hinweist. ELISA Untersuchungen zeigten, dass Wt- und Pax4+ Zellen in Abhängigkeit von der Glucose-Konzentration Insulin abgeben. Pax4+ Zellen zeigten einen höheren intrazellulären Insulingehalt, aber das Verhältnis zwischen freigesetztem und intrazellulärem Insulingehalt war in Wt- und Pax4+ Zellen annähernd gleich. Der höhere intrazelluläre Insulingehalt und die erhöhte Insulinabgabe von Pax4+ Zellen korrelierte mit einer höheren C-Peptid-Expression im Vergleich zu Wt-Zellen [3].

Das *in vivo* Potenzial der differenzierten Wt- und Pax4+ Zellen wurde in Transplantations-Experimenten mit Streptozotocin (STZ)-induzierten diabetischen Mäusen getestet. Die Zellen wurden in die Nierenkapsel und in die Milz hyperglykämischer Mäuse transplantiert. Mäuse, die mit Wt-Zellen transplantiert waren und Kontrolltiere entwickelten einen hyperglykämischen Blutzuckerwert von 25-30 mM und litten an diabetischem Gewichtsverlust. In den mit Pax4+ Zellen transplantierten Tieren war der Blutzuckerspiegel 5 Wochen nach der Transplantation wieder normalisiert. Ebenso konnte der Gewichtverlust in mit Pax4+-transplantierten Mäusen aufgehoben werden. Partielle Nephrektomie führte zu einem erneuten Anstieg des Blutzuckerspiegels [3]. Der Einsatz des neuen Protokolls zeigte, dass die Selektion Nestin-positiver Zellen weder notwendig noch vorteilhaft für die Entwicklung von Insel-ähnlichen Clustern aus ES Zellen ist.

Charakterisierung pankreatischer Vorläuferzellen

In diesen Untersuchungen wurde das neue Differenzierungsmodell eingesetzt und differenzierende Wt- und Pax4+ Zellen wurden vor Beginn, in einem intermediären Stadium und am Ende der pankreatischen Differenzierung analysiert. Immunfluoreszenz-Analyse zeigte, dass Nestin und C-Peptid im intermediären Stadium in pankreatischen Vorläuferzellen

nachweisbar waren, während im terminalen Differenzierungsstadium die C-Peptid-positiven Insel-ähnlichen Cluster Nestin-negativ sind. Das deutet auf eine transiente Expression von Nestin während der pankreatischen Differenzierung hin [3]. Ein weiteres Intermediärfilamentprotein, Desmin, zeigte Ko-Expression mit Nestin im Vorläuferstadium, jedoch nicht in den pankreatischen Vorläuferzellen, was darauf hinweist, dass Desmin bei der Differenzierung von Pankreaszellen keine Rolle spielt [3].

Cytokeratin 19 (CK19) mRNA wurde in allen analysierten Differenzierungsstadien nachgewiesen, jedoch ließ sichCK19 nach pankreatischer Differenzierung nicht mehr in der Intermediärfilamentstruktur, sondern nur als unorganisiertes (dot-like) Protein nachweisen. Immuofluoreszenzanalyse ergab eine hohe Ko-Expression von CK19 mit Nestin und C-Peptid im pankreatischen Vorläuferstadium. Im terminalen Stadium waren einige C-Peptid-positive Zellen aus Insel-ähnlichen Clustern CK19-negativ, was ein Hinweis auf eine zumindest teilweise Reifung der pankreatischen Zellen ist [3]. Dagegen war Isl-1 in den Nestin-positiven Subpopulationen nur in pankreatischen Vorläuferzellen, nicht aber in differenzierten Insulin-positiven Zellen nachweisbar [3].

Die Untersuchung der Expression von Karbonsäureanhydrase II (CaAII), einem Protein des duktalen Pankreas-Epithels, ergab einen hohen Grad der Ko-Expression mit C-Peptid im pankreatischen Vorläuferstadium und im terminalen Entwicklungsstadium. Diese Ergebnisse lassen vermuten, daß es sich bei den *in vitro* entwickelten Zellen um noch unreife pankreatische Zellpopulation handelt, da CaAII in reifen pankreatischen Beta-Zellen herunter reguliert ist [3].

Die Ergebnisse zeigen, dass die Gene, die bei der Entwicklung und Funktion von pankreatischen Beta-Zellen *in vivo* eine Rolle spielen, auch während der ES-Zell-Differenzierung in die pankreatische Linie *in vitro* ausgebildet werden. Weiterhin konnte gezeigt werden, dass das Intermediärfilamentprotein Nestin während der Entwicklung von ES-Zellen in Insel-ähnliche Cluster transient gebildet wird.

Schlußfolgerungen

Unsere Studien haben gezeigt, daß ES Zellen fähig sind, sich *in vitro* in Insel-ähnliche Cluster zu entwickeln. Die Zellen sind charakterisiert durch die Expression pankreasspezifischer Gene, Beta-Zell-spezifischer Ionenkanal-Aktivität, Glucose abhängiger Insulinabgabe und die Fähigkeit, den Blutzuckerspiegel diabetischer Mäuse zu normalisieren.

Die Expression des pankreasspezifischen Entwicklungskontrollgen Pax4 in ES-Zellen hatte einen positiven Effekt auf die Differenzierung und teilweise Reifung von pankreatischen Zellen. Unsere Ergebnisse weisen darauf hin, dass Pax4 eine entscheidende Rolle bei der Kontrolle der Entwicklung undifferenzierter ES Zellen in die pankreatische Linie spielt und in Differenzierungsstrategien mit adulten Stammzellen einbezogen werden sollte.

Die transiente Expression von Nestin während der pankreatischen Differenzierung *in vitro* (ohne die Selektion von Nestin positiven Vorläuferzellen) weist auf eine mögliche Bedeutung während der pankreatischen Differenzierung hin, lässt aber gleichzeitig vermuten, dass Nestin *in vivo* und in *in vitro*-Systemen unterschiedlich exprimiert wird. Unsere Arbeit zeigt, dass die Entwicklung von ES-Zellen in funktionelle pankreatische Zellen möglich ist. Dies eröffnet die Entwicklung neuer Strategien für die Erzeugung Insulin-produzierender Zellen für den therapeutischen Einsatz für die Behandlung von Diabetes. Jedoch müssen vor der therapeutischen Anwendung alle Probleme bezüglich der Reifung und der Reinheit der aus ES-Zellen generierten Spenderzellen beantwortet werden. Darüberhinaus kommt der Identifizierung von wichtigen Signalmolekülen und Differenzierungsfaktoren, die innerhalb der Inselzellen für die Entwicklung und Funktion von Beta-Zellen verantwortlich sind, eine entscheidende Bedeutung zu.

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12. Reprints of publications and manuscripts on which thesis is based

[1] - Blyszczuk P, Czyz J, Kania G, Wagner M, Roll U, St-Onge L, Wobus AM. (2003) Expression of Pax4 in embryonic stem cells promotes differentiation of nestin-positive progenitor and insulin-producing cells. *Proc. Natl. Acad. Sci. USA*, 100, 998-1003

[2] - Blyszczuk P and Wobus AM. (2004) Stem cells and pancreatic differentiation *in vitro*. *J. Biotechnology,* 113 (1-3), 3-13

[3] - Blyszczuk P, Asbrand C, Rozzo A, Kania G, St-Onge L, Rupnik M and Wobus AM. Generation of functional beta-like cells from embryonic stem cells and characterization of pancreatic progenitor cells expressing nestin *Int. J. Dev. Biol.* (in press)

[4] - Blyszczuk P, Kania G and Wobus AM. Pancreatic and hepatic ES cell differentiation *in vitro*. Chapter 9 in *Embryonic stem cells – a practical approach.* Eds. E. Notarianni and M. Evans, *Oxford University Press*. (in press)

[5] - Kania G, Blyszczuk P, Czyz J, Navarrete-Santos A, Wobus AM. (2003) Differentiation of mouse embryonic stem cells into pancreatic and hepatic cells. *Methods in Enzymology* 365, 287-303

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Expression of Pax4 in embryonic stem cells promotes differentiation of nestin-positive progenitor and insulin-producing cells

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Mouse embryonic stem (ES) cells differentiate into cells of all three primary germ layers including endodermal cells that produce insulin *in vitro*. We show that constitutive expression of Pax4 (Pax4$^+$), and to a lesser extent Pdx1 (Pdx1$^+$), affects the differentiation of ES cells and significantly promote the development of insulin-producing cells. In Pax4 overexpressing R1 ES cells, isl-1, ngn3, insulin, islet amyloid polypeptide, and glucose transporter 2 (Glut-2) mRNA levels increase significantly. The number of nestin-expressing (nestin+) cells also increases. Constitutive Pax4 expression combined with selection of nestin+ cells and histotypic culture conditions give rise to spheroids containing insulin-positive granules typical of embryonal and adult β cells. In response to glucose, Pax4$^+$ and wild-type ES-derived cells release insulin. Transplantation of these cells into streptozotocin-treated diabetic mice results in a normalization of blood glucose levels. We conclude that constitutive expression of Pax4 in combination with histotypic cultivation facilitates ES cell differentiation into the pancreatic lineage, which leads to the formation of islet-like spheroid structures that produce increased levels of insulin.

Transplantation of pancreatic islet cells is a promising therapeutic option for the treatment of diabetes. However, the lack of suitable donor tissues remains a major obstacle. Transplantation of adult or fetal islets of Langerhans as a therapy for insulin-dependent diabetes is limited because of the low availability of human donor pancreas (1). Pancreatic stem cells residing within the ductal epithelium have been used to generate mouse and human islet-like clusters (2, 3), which partially reverted insulin-dependent diabetes in animal models (3), but low proliferation rates may limit its wide scale application.

An alternative source for the generation of insulin-producing cells are embryonic stem (ES) cells which have almost unlimited proliferation capabilities while retaining the potential to differentiate *in vitro* into cells of the three primary germ layers (4, 5). ES cells of mice (6, 7) and human (8) have been shown to differentiate into insulin-secreting cells and to normalize blood glucose levels when transplanted into diabetic mouse models (6). Because this approach may be inefficient and generated in most cases only low amounts of insulin-producing cells, directing ES cells toward the β cell lineage would most likely improve their formation.

During embryogenesis, several growth and transcription factors are involved in β cells differentiation (for review, see ref. 9). Pdx1 and Pax4 are essential for proper β cell development. Pdx1 is expressed throughout the epithelium of early pancreatic buds and becomes restricted to β cells in the adult animal where it plays a role in insulin expression and glucose response (10). Pdx1-mutant mice do not develop any pancreas (11, 12). Pax4 is restricted to the β and δ cell lineages, and mice lacking Pax4 fail to develop any β cells and are diabetic (13).

Pancreatic progenitor cells are at present poorly defined. The four endocrine cell types arise from a common progenitor cell residing within the epithelium of the pancreatic bud. In adult animals, pancreatic stem cells are thought to reside in the exocrine duct system and within the islet itself. Recently, cells expressing the neurofilament protein nestin are proposed to represent multipotent progenitor cells of the pancreas (14, 15), and on special cultivation, insulin-producing cells have been derived *in vitro* from nestin-positive cells (7, 15).

In the present study, we analyzed the influence of constitutive expression of Pdx1 and Pax4 genes on ES cell differentiation into pancreatic cells and developed protocols for efficient generation of insulin-producing cells. We show that activation of Pax4 expression in ES cells and selective differentiation via nestin+ progenitor cells followed by histotypic culture increases the amount of insulin produced by the cells.

Materials and Methods

Generation of Vectors and Cell Lines. The mouse Pdx1 or Pax4 gene was placed under the control of the cytomegalovirus (CMV) early promoter/enhancer region by inserting the complete cDNA of each gene into the vector pACCMV.pLpA (16). Mouse ES cells of line R1 [wild type (wt)] (17) were coelectroporated (18) with CMV-Pdx1 or CMV-Pax4 and the neomycin resistance gene under the control of the phosphoglycerate kinase I promoter [pGKneo (19)].

Cell Culture and Differentiation Conditions. The wt cells and cells constitutively expressing Pdx1 (Pdx1$^+$) and Pax4 (Pax4$^+$) were cultivated on feeder layer of mouse embryonic fibroblasts in the presence of recombinant human leukemia inhibitory factor as described (5). Pdx1$^+$ and Pax4$^+$ cells were additionally supplemented with 300 ng/ml G418.

For differentiation ("basic" protocol), wt, Pdx1$^+$, and Pax4$^+$ ES cells ($n = 600$) were used to form embryoid bodies (EBs) that were cultivated in hanging drops as described (5). At day 5, EBs were plated in Iscove's modified Dulbecco's medium (IMDM, GIBCO) supplemented with 20% FCS, L-glutamine, nonessential amino acids, and α-monothioglycerol (see ref. 5).

Alternatively, wt and Pax4$^+$ ES cells ($n = 200$) were cultivated as EBs in hanging drops and plated at day 4, and nestin-positive cells were selected by the "nestin+ selection" protocol (see ref. 20). At day 4 + 8, EB outgrowths were dissociated by 0.1% trypsin (GIBCO)/0.08% EDTA (Sigma) (1:1) in PBS for 1 min, collected by centrifugation, and replated onto poly-L-ornithine/laminin-coated tissue culture dishes in DMEM/F12 (GIBCO) containing 20 nM progesterone, 100 μM putrescine, 1 μg/ml laminin, 10 mM nicotinamide, 25 μg/ml insulin, 30 nM sodium selenite (all from Sigma), 50 μg/ml transferrin, 5 μg/ml fi-

Abbreviations: EB, embryoid body; ES, embryonic stem; Glut-2, glucose transporter 2; LI, labeling index; STZ, streptozotocin; CMV, cytomegalovirus; wt, wild type.

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bronectin, B27 media supplement (all from GIBCO), and 20% FCS (7). At day 4 + 9, FCS was removed.

Estimation of mRNA Levels by Semiquantitative RT-PCR Analysis. Total cellular mRNA of wt, Pdx1$^+$, and Pax4$^+$ ES cells, EBs or EB outgrowths were reverse transcribed, amplified by PCR, electrophoretically separated, and analyzed as described (5). For semiquantitative determination of mRNA levels of the candidate genes [shh, isl-1, Pdx1, ngn3, Pax6, insulin, islet amyloid polypeptide, glucose transporter 2 (Glut-2), Pax4, and β-tubulin, primer sequences available on request], transcript levels were standardized to the corresponding β-tubulin level, and for each candidate gene, mRNA levels relative to the highest candidate gene level were estimated in percentage (5).

Immunofluorescence and Quantitative Immunoassay. Cells were fixed with 4% paraformaldehyde processed for immunofluorescence microscopy as described (5). The following primary Abs were used: mouse anti-insulin (Sigma), rabbit anti-glucagon, rabbit anti-PP, rabbit antisomatostatin (all from Dako), and mouse antinestin (clone rat 401, Developmental Studies Hybridoma Bank, Iowa City). Cy^3-conjugated goat anti-mouse IgG and fluorescein (DTAF)-conjugated goat anti rabbit IgG (both from Jackson ImmunoResearch) were used as secondary Abs. Samples were analyzed by the fluorescence microscope ECLIPSE TE300 (Nikon) and the confocal laser scanning microscope LSM-410 (Zeiss). Because insulin- and nestin-positive cells were predominantly found in multilayered, compacted clusters and localized to different structures (cytoplasm or intermediate filament proteins), the percentage values of immunolabeled cells could be estimated only in arbitrary units. Therefore, a semiquantitative immunofluorescence imaging analysis was performed by using the LUCIA M, version 3.52a software (Nikon). Randomly selected ($n = 25$) but representative areas of each sample were analyzed for the labeling index (LI) defined by the ratio of the positive signal area to the measured area.

Quantitative Insulin Determination by ELISA. To estimate total cellular and secreted insulin levels, differentiated wt and Pax4$^+$ cells were preincubated for 90 min at 37°C in Krebs Ringer Bicarbonate Hepes buffer supplemented with 2.5 mM glucose. For induced insulin release, cells were further incubated in Krebs Ringer Bicarbonate Hepes buffer supplemented with 27.7 mM glucose and alternatively with 5.5 mM glucose and 10 μM tolbutamide (Sigma) for 15 min at 37°C. The control was incubated with 5.5 mM glucose. Proteins were extracted from the cells with acid ethanol at 4°C overnight, followed by cell sonification. Determination of cellular and secreted insulin was performed by using an insulin ELISA kit (Mercodia, Uppsala). Protein was determined by the Bradford assay (Bio-Rad).

Histotypic Maturation into Spheroids. The wt and Pax4$^+$ cell clusters cultivated according to the nestin+ selection protocol at stage 4 + 28 d were dissociated by 0.1% trypsin/0.08% EDTA in PBS (1:1) for 1 min, collected by centrifugation and transferred into 6 cm bacteriological plates in the medium described for nestin+ selection at stage 4 + 9 d. After overnight culture in suspension, spheres were transferred into 100-ml "Spinner" flasks and cultured in the CELLSPIN system (Integra Bioscience, Wallisellen, Switzerland) at 30 rpm agitation at 37°C up to 10 d.

For immunohistochemical detection of insulin and glucagon, spheroids were fixed in Bouin solution, embedded in paraffin, sectioned at 5 μm by conventional techniques, and immunolabeled as described above.

Immunogold Labeling and Electron Microscopy. Spheroids were fixed in 0.1% glutaraldehyde/4% formaldehyde in 0.1 M cacodylate buffer, transferred to 0.1 M cacodylate buffer, and embedded. Ultrathin sections were sequentially treated with 10% H_2O_2, washed with 0.9% NaCl, blocked with 3% BSA, incubated overnight with the primary Ab in PBS supplemented with 0.5% BSA (rabbit anti-porcine insulin, 1:50, ICN), washed with PBS, and reblocked with goat serum. Binding of the primary Ab was visualized with gold labeled secondary Ab (15-nm gold particles, goat–anti-rabbit, Amersham Pharmacia), and electron-microscopy was performed after contrasting the sections. Tissue samples of adult murine pancreas (control) were fixed and processed as described.

Transplantation into Streptozotocin (STZ)-Diabetic Mice. Nonfasted 8- to 10-wk-old male BALB/c mice were treated with 200 mg/kg body weight STZ (Sigma) freshly dissolved in 0,025 M tri-sodium citrate 2-hydrate (pH 4.0). Mice were anaesthetized by i.p. injection of 15 μl/g body weight avertin (2.5% tribromoethyl alcohol:tertiary amyl alcohol). The left kidney and the spleen were exposed through a lumbar incision and cells were transferred into each tissue by using a blunt 30-gauge needle (Hamilton). Between 1×10^6 and 2×10^6 cells were transplanted per animal 24–48 h after STZ treatment. Blood glucose was measured daily between 9:00 and 11:00 a.m. from snipped tail by using a OneTouch FastTake blood glucose monitoring system (LifeScan, Mountain View, CA). Recipient animals were killed by cervical dislocation 14 d after STZ treatment, and the kidney and spleen were analyzed by immunohistochemistry on paraffin sections for the presence of insulin-producing cells (13).

Results

Pax4 Activation in ES Cells Modified the Differentiation Pattern and Expression Levels of Pancreatic Genes. To assess whether developmental control genes expressed during pancreas development can enhance the differentiation of ES cells into insulin-producing cells, we generated ES cell lines expressing the Pdx1 or Pax4 gene under the control of the human CMV early promoter/enhancer region. After EB formation, no differences were observed in the diameter of 5-d-old EBs (between 0.44 and 0.49 mm) derived from wt, Pdx1$^+$, and Pax4$^+$ cells, suggesting that transgene expression did not affect ES cell proliferation within the EB. Constitutive expression of Pdx1 modestly affected the ES cell differentiation pattern; however, significant differences were found in Pax4$^+$-derived cells. Cardiac differentiation was delayed and reduced, and the degree of skeletal and neuronal differentiation was significantly lower in Pax4$^+$ cells in comparison to wt cells (not shown).

The morphological studies were confirmed by RT-PCR analyses of pancreatic developmental control genes (Fig. 1). Important changes in the expression levels of those genes were detected in Pax4$^+$ and, to a lesser extent, Pdx1$^+$ cells. No differences in shh mRNA levels were found among wt, Pdx1$^+$, and Pax4$^+$ cells. For isl-1, likewise, we detected few differences in expression between wt and Pdx1$^+$ cells, whereas Pax4$^+$-derived cells displayed a maximum mRNA level at differentiation stage 5 + 10 d. As expected, Pdx1$^+$ cells showed 4- to 5-fold higher Pdx1 levels at all stages when compared to wt and Pax4$^+$ cells. Ngn3 mRNA levels were up-regulated at early and intermediate stages in both Pdx1$^+$ and Pax4$^+$ cells. Pax6 levels were significantly higher in Pdx1$^+$-derived cells at all stages, whereas in Pax4$^+$ cells, Pax6 levels were up-regulated during early differentiation stages but significantly down-regulated during later stages. Low insulin mRNA levels were found in undifferentiated wt, Pdx1$^+$, and Pax4$^+$ cells, but after differentiation, insulin expression increased in all cell lines with maximum levels measured in Pax4$^+$ cells. Maximum levels of islet amyloid polypeptide and Glut-2 mRNA were recorded only in Pax4$^+$ cells at a late stage. High Pax4 transcript levels were measured in

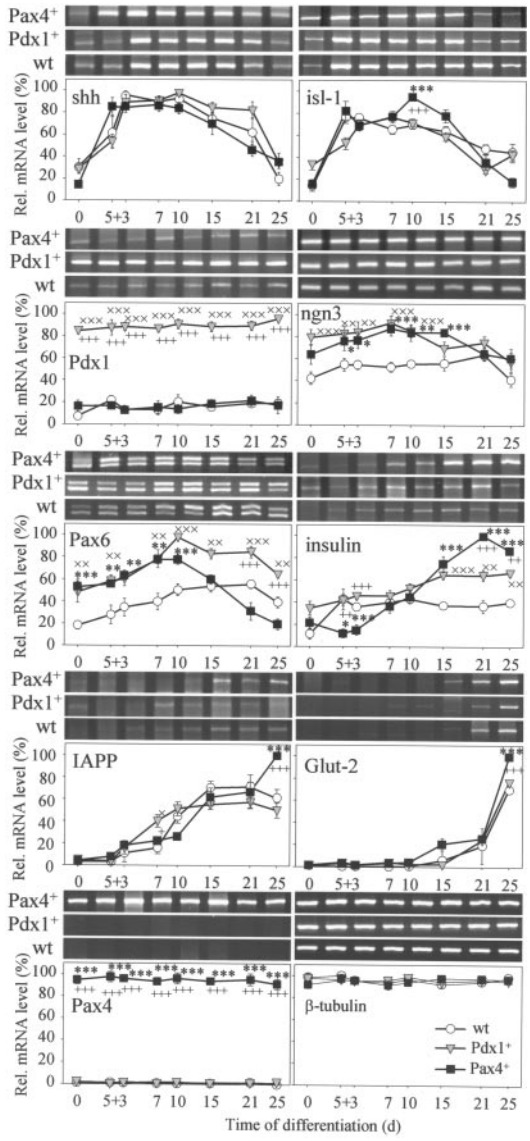

Fig. 1. RT-PCR analysis of wt-, Pdx1$^+$-, and Pax4$^+$-derived cells cultivated according to the basic protocol. Sonic hedgehog (shh), islet-1 (isl-1), Pdx1, neurogenin3 (ngn3), Pax6, insulin, islet amyloid polypeptide (IAPP), Glut-2, Pax4, and β-tubulin mRNA levels were determined. Each value (n = five experiments) represents mean ± SEM. Statistical significance was tested between wt and Pdx1$^+$ (×), wt and Pax4$^+$ (*), and Pdx1$^+$ and Pax4$^+$ (+) cells, respectively, by Student's t test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

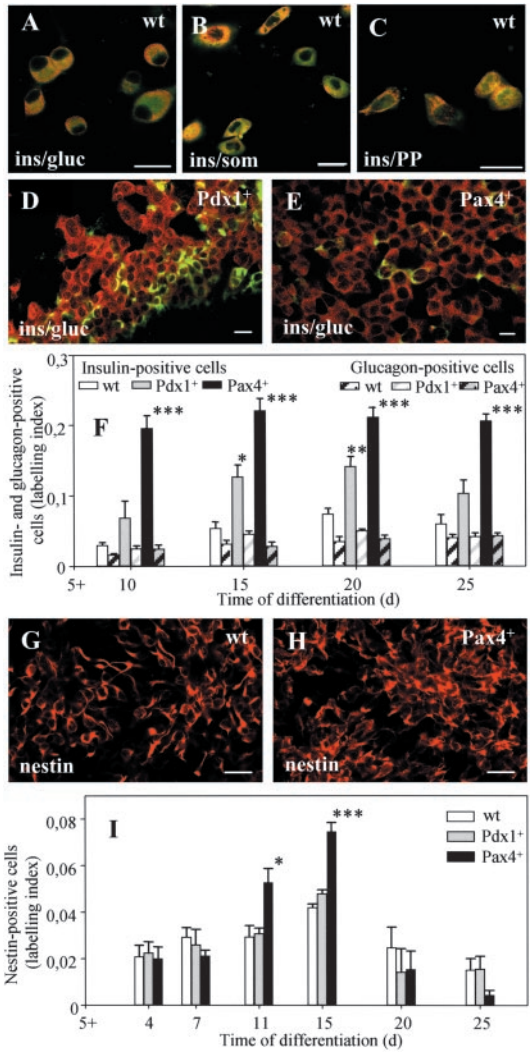

Fig. 2. Immunofluorescence analysis of pancreatic proteins (*A–F*) and of nestin (*G–I*) of wt- (*A–C* and *G*), Pdx1$^+$- (*D*), and Pax4$^+$ (*E* and *H*)-derived cells differentiated according to the basic protocol. Shown are wt cells expressing insulin and glucagon (ins/gluc, *A*), insulin and somatostatin (ins/som, *B*), and insulin and pancreatic polypeptide (ins/PP, *C*), and shown are Pdx1$^+$ (*D*), and Pax4$^+$ (*E*) cells expressing insulin or glucagon (ins/gluc) at days 5 + 15 to 5 + 20. Semiquantitative immunofluorescence analysis was done to estimate the labeling index of insulin and glucagon (*F*) and of nestin (*I*) in wt, Pdx1$^+$, and Pax4$^+$ cells. Each value of $n = 5$ (*F*) and $n = 4$ (*I*) experiments represents mean ± SEM. Statistical significance was tested by Student's t test: *, $P < 0.05$; **, $P < 0.01$, ***; $P < 0.001$. (Bar = 20 μm.)

Pax4$^+$ cells; however, no detectable transcript levels were found in wt and Pdx1$^+$ cells (Fig. 1).

Pax4 Expression Increased the Number of Insulin- but not Glucagon-Producing Cells. To investigate the expression of the pancreatic hormones insulin, glucagon, somatostatin, and PP in wt, Pdx1$^+$, and Pax4$^+$ cells, immunofluorescence analyses were performed. The wt ES cells spontaneously differentiated into cells predominantly coexpressing all four pancreatic hormones (Fig. 2 *A–C*), whereas few cells were positive for insulin only. The amount of insulin-positive cells was estimated by the LI. The wt cells showed a LI of 0.074 corresponding to ≈10–15% insulin-positive cells (Fig. 2*F*). No significant changes were detected in the proportion of insulin- vs. glucagon-positive cells during all differentiation stages (Fig. 2*F*). In Pdx1$^+$ cells, a higher number of insulin-positive cells was observed (LI: 0.12) corresponding to ≈20% of the total cell population (Fig. 2 *D* and *F*).

In Pax4$^+$ cells, the amount of insulin-positive cells was significantly higher with a LI of 0.24 corresponding to ≈60% insulin-positive cells that were mainly organized in compact clusters (Fig. 2*E*). Pax4$^+$ cells revealed a constant 4.5-fold increase throughout the differentiation period, whereas the level of glucagon-positive cells remained constant at all stages (Fig. 2*F*), indicating that Pax4$^+$ cells differentiated mainly into cells producing only insulin.

Pax4 Expression Resulted in an Increase in the Number of Nestin-Positive Cells. Nestin expression has been used to select ES-derived cells capable of differentiation into insulin-producing cells *in vitro* (7). Consequently, we performed a comparative analysis of nestin expression in wt, Pdx1$^+$, and Pax4$^+$ ES cells

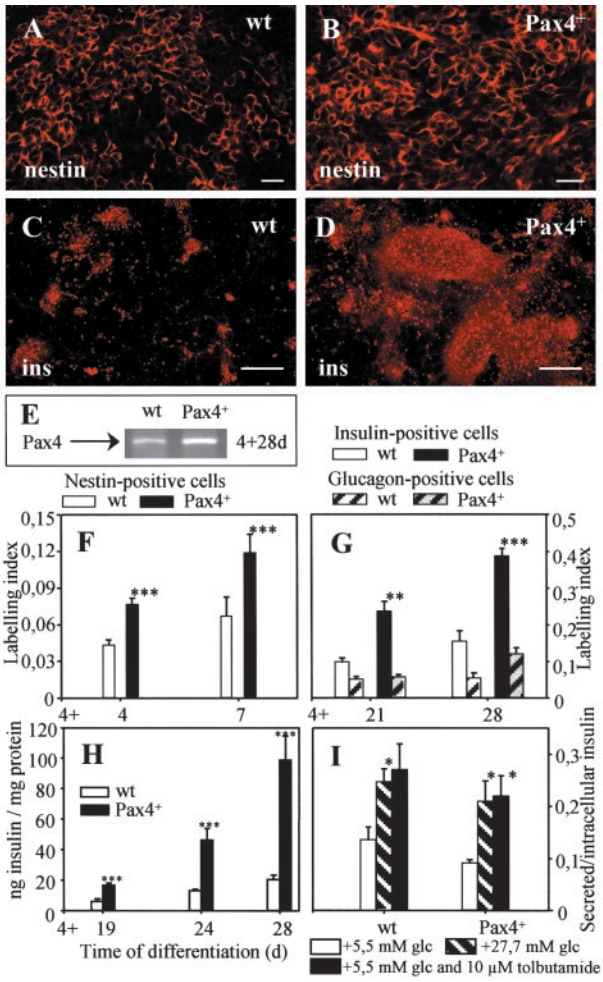

Fig. 3. Immunofluorescence (*A–D*) and semiquantitative imaging analysis (*F* and *G*) of nestin (*A*, *B*, and *F*) and insulin (ins, *C*, *D*, and *G*) labeling of wt- (*A* and *C*) and Pax4$^+$ (*B* and *D*)-derived cells at days 4 + 7 (*A*, *B*, and *F*; $n = 4$) and 4 + 28 (*C*, *D*, and *G*; $n = 3$, with two parallels) differentiated according to the nestin+ selection protocol. (*E*) Pax4 mRNA level after differentiation via the nestin+ selection protocol at day 4 + 28 in wt and Pax4$^+$ cells. Intracellular insulin levels (*H*, $n = 5$) and glucose-induced insulin release (*I*, $n = 3$) of wt- and Pax4$^+$-derived cells were determined by ELISA. Insulin release expressed by the ratio of secreted to intracellular insulin levels (*I*) was measured in response to 27.7 mM glucose (striped), 5.5 mM glucose with 10 μM tolbutamide (black) in wt and Pax4$^+$ cells after 15 min of static incubation; 5.5 mM glucose was used as control (white). Each value represents mean ± SEM. Statistical significance was tested by Student's *t* test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. [Bar = 20 μm (*A* and *B*) and 200 μm (*C* and *D*).]

after differentiation according to the basic protocol (Fig. 2 *G–I*). Pax4$^+$ cells showed a significant increase in the number of nestin+ cells at stages 5 + 11 d and 5 + 15 d (Fig. 2*H*) when compared to wt (Fig. 2*G*) or Pdx1$^+$ (not shown) cells (Fig. 2*I*).

In subsequent experiments, wt and Pax4$^+$ ES cells were differentiated under culture conditions selective for the development of nestin-expressing cells [nestin+ selection protocol (20)]. An acceleration and increase in the amount of nestin-positive cells (Fig. 3 *A* and *B*) was found when compared to the results obtained with the basic protocol (Fig. 2*I*). Differentiated Pax4$^+$ cells generated a higher number of nestin+ cells at an earlier differentiation stage (compare LI at stages 5 + 4 and 5 + 7 d of Fig. 2*I* with stages 4 + 4 and 4 + 7 d of Fig. 3*F*).

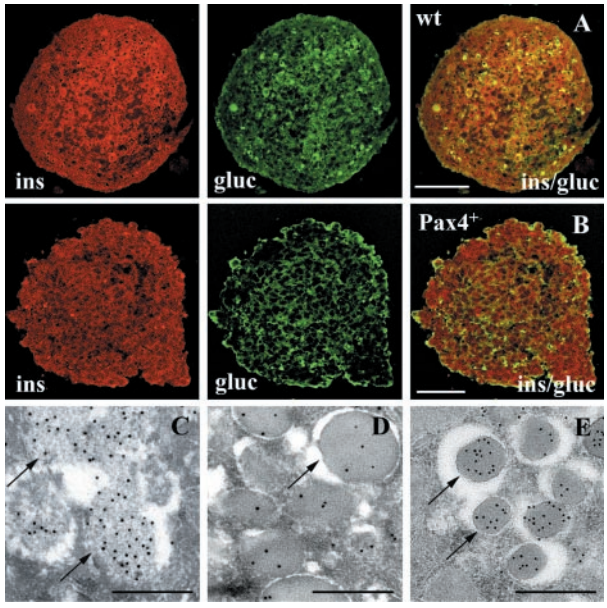

Fig. 4. Immunofluorescence (*A* and *B*) and electronmicroscopical (*C–E*) analysis of wt- (*A*) and Pax4$^+$ (*B–D*)-derived spheroids generated by nestin+ selection and 10-d differentiation in histotypic culture. Shown are insulin- (red) and glucagon (green)-labeled sections of Pax4$^+$ spheroids with faint globular structures (*C*, arrow) filled with floccular low-density material labeled with gold grains indicating insulin. More differentiated Pax4$^+$ cells showed cytoplasmic granules with an electron dense core (*D*) in comparison to insulin-labeled secretory granules of β cells of adult mouse pancreas (*E*). [Bar = 50 μm (*A* and *B*) and 0.5 μm (*C—E*).]

Differentiation of Pax4$^+$ Cells According to the Nestin+ Selection Protocol Resulted in an Increase of Intracellular Insulin Levels. By applying culture conditions selective for the development of nestin+ cells (20) followed by induction of pancreatic differentiation (7), Pax4$^+$ ES cells resulted in a considerable increase in the number of insulin-producing cells (Fig. 3*D*) in comparison to wt cells (Fig. 3*C*). Differentiation of wt cells according to the nestin+ selection protocol resulted in significant Pax4 transcript levels at stage 4 + 28 d (compare Fig. 3*E* to Fig. 1).

Imaging analysis of immunofluorescence signals confirmed that Pax4 expression significantly increased the LI (0.39 corresponding to 80% insulin-positive cells), whereas wt cells resulted in a lower LI of 0.15 equivalent to ≈20–25% insulin-positive cells (Fig. 3 *C*, *D*, and *G*). No significant differences in glucagon-labeling were observed between wt and Pax4$^+$ cells (Fig. 3*G*).

Intracellular insulin levels analyzed by ELISA were significantly increased in Pax4$^+$ cells (98.7 ng insulin/mg protein), whereas wt cells showed only a moderate increase (20.7 ng insulin/mg protein) at stage 4 + 28 d (Fig. 3*H*). To determine, whether wt and Pax4$^+$-derived cells were glucose-responsive, we analyzed insulin release in the presence of low (5 mM) and high glucose (27.7 mM) concentration. Both, wt and Pax4$^+$ cells secreted insulin in response to glucose at an advanced stage of 4 + 32 d (but not at 4 + 28 d), suggesting a maturation effect during differentiation. wt and Pax4$^+$ cells also responded to tolbutamide, a sulfonylurea known to stimulate insulin secretion (Fig. 3*I*).

Histotypic Generation of Spheroids and Ultrastructural Analysis of Insulin-Producing Cells. Cells generated according to the nestin+ selection protocol for 4 + 28 d followed by 10-d histotypic "Spinner" cultivation showed significant accumulation of insulin and glucagon in spheroids (Fig. 4 *A* and *B*). The intracellular

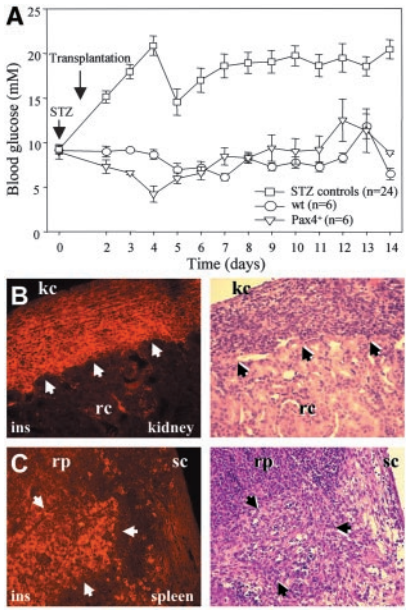

Fig. 5. Transplantation of wt and Pax4$^+$ insulin-producing cells into STZ-treated diabetic mice. Shown are blood glucose levels in control STZ mice that did not receive any cells (control) and mice transplanted with wt or Pax4$^+$ cells (*A*), and the immunofluorescence analyses by using an insulin-specific Ab of kidney (*B*) and spleen (*C*) of mice transplanted with Pax4$^+$ cells. Arrowheads in hematoxylin/eosin stainings define the area of transplanted insulin-positive cells in kidney and spleen. kc, kidney capsule; rc, renal cortex; rp, red pulp; and sc, splenic capsule.

insulin levels amounted to 297.4 ± 18.0 (wt) and 455.5.6 ± 64.8 (Pax4$^+$) ng/mg protein.

Immune electronmicroscopy of Pax4$^+$ cells showed numerous insulin-positive cells (Fig. 4 *C* and *D*). Immunogold-labeling detected faint globular structures of different size filled with floccular low density material (Fig. 4*C*) and some granules with an electron dense core at the apical pole of the cells (Fig. 4*D*). The insulin-positive granules were of comparable size to secretory granules of β cells of the adult murine pancreas (Fig. 4*E*); however the labeling density was lower in Pax4$^+$ derived spheroids (Fig. 4*D*) as compared to adult β cells (Fig. 4*E*). Differentiated wt cells did not show any secretory granules and only occasionally cytoplasmic insulin labeling (not shown).

Transplantation of ES Cell-Derived Insulin-Producing Cells Maintained Glucose Homeostasis in Diabetic Animals. The potential of wt- and Pax4$^+$-derived insulin-producing cells to maintain normal glucose homeostasis was investigated by transplanting the cells into STZ-induced diabetic mice. Between 1 and 2 million cells were transplanted under the kidney capsule and into the spleen. Mice transplanted with wt or Pax4$^+$ cells (differentiated according to the nestin+ selection protocol) 24 h after STZ treatment retained normal blood glucose levels (i.e., below 10 mmol/liter) over a period of 14 d (Fig. 5*A*). Nontransplanted control animals became hyperglycemic 48 h after STZ treatment, with blood glucose levels >10 mmol/liter. Recipient animals were then killed and the transplanted cells were analyzed by immunohistology and conventional hematoxylin/eosin staining. Numerous insulin-positive cells were observed under the kidney capsule (Fig. 5*B*) and in the spleen (Fig. 5*C*) of animals transplanted with Pax4$^+$ (Fig. 5 *B* and *C*) and wt (not shown) cells.

Discussion

Whereas recent studies have shown the generation of insulin-producing cells from progenitor cells of the pancreas (2, 3), liver (21), and pluripotent ES cells of mouse (6, 7) and human (8) origin, the efficiency of *in vitro* generated insulin-producing cells is low and presently insufficient for large scale therapeutic applications.

To overcome this limitation, we devised a strategy to induce the formation of insulin-producing cells by overexpressing the Pax4 gene in ES cells. We show here that constitutive expression of Pax4 revealed a significant up-regulation of genes involved in β-cell development and function resulting in an increased number of insulin-positive cells and in the amount of insulin being produced. This increase in the efficiency of Pax4$^+$ cells to direct undifferentiated ES cells into insulin-producing cells was seen in three different culture systems: the basic and nestin+ selection protocol, and a histotypic culture model. Pax4 overexpression also resulted in an increase in the differentiation status of the cells, as shown by ultrastructural analyses and by a higher proportion of cells producing insulin only, whereas wt cells differentiated mainly into cells coexpressing both insulin and glucagon.

The RT-PCR data showed no effects of Pax4 and Pdx1 expression on shh, isl-1, and Pdx1 transcript levels, genes affecting early pancreatic differentiation. However, expression levels of genes controlling endocrine precursor specification, such as ngn3 and Pax6 (22, 23), were up-regulated in Pax4$^+$ (and Pdx1$^+$) cells. The early activation of Pax6 transcript levels, but down-regulation at later stages in Pax4$^+$ cells could be explained by specific regulatory functions of Pax4 on α- vs. β-cell specification. However, Pax6, as well as ngn3 and isl-1, are also significantly expressed during neuronal differentiation and specification (24–26) indicating that the expression of these genes may be associated with other endodermal or neuroectodermal cell types present in differentiating EBs.

The results suggested that Pax4 may play a significant role in directing undifferentiated ES cells into endocrine insulin-producing cells, but low insulin levels were obtained in all cell lines differentiated according to the basic protocol. However, when a differentiation procedure selecting for potential nestin+ precursor cells was applied (7), Pax4$^+$ cells showed an increase in the formation of insulin-producing cells from ≈60% (basic protocol) to 80% (nestin+ selection) compared to 10–15% (basic protocol) and 20–25% (nestin+ selection) insulin-producing cells generated from wt cells. As a consequence, a fivefold increase of the intracellular insulin level was detected in Pax4$^+$ cells.

This finding of increased differentiation efficiency of insulin-producing cells under conditions that selected for a nestin+ cell population (7) raised several questions. Although, nestin+ cells may participate in the neogenesis of endocrine islet cells (14, 15), recent results (27) showed that, *in vivo*, nestin is expressed in mesenchymal and not in epithelial cells, where endocrine progenitor cells reside.

The role of nestin expression and the significance of nestin+ cells as a potential progenitor cell type is not yet known. Nestin is transiently expressed in different cell types of embryonic and adult tissues and is suggested to play a transient role in proliferation and migration processes of progenitor cells (28). One might speculate that nestin+ cells are characterized by a high developmental plasticity and represent a common progenitor cell population which *in vitro* under the influence of genetic and/or epigenetic factors can be programmed either into a neural (29), hepatic (21), or pancreatic endocrine (7, 15) fate. Previous findings would support this hypothesis: The expression of transcription factors ngn3, isl-1, Pax6, and Pax4, and of neuropeptide-processing enzymes were reported both, for cultured neuronal (29) and pancreatic endocrine cells (30).

In parallel to the selective differentiation via nestin+ cells, insulin levels could be further enhanced when cells were additionally differentiated in a histotypic culture system as 3D

spheroids. Obviously, communication via cell–cell interactions is necessary to support cell-type-specific differentiation *in vitro* as demonstrated for liver, retinal, and pancreatic cells (31, 32). Spheroids generated from wt and Pax4$^+$ ES cells showed insulin- and glucagon-producing cells in a histotypic organization. Similarly, reaggregation of human fetal islet cells increased the differentiation state (33).

The role of histotypic differentiation of ES-derived pancreatic cells with respect to cellular specification was further supported by ultrastructural analysis. Spheroids showed an intracellular accumulation of insulin, and Pax4$^+$ clusters revealed similar granular structures as described for embryonic β cells (34). Although, the density of insulin labeling in the secretory granules of Pax4$^+$ cells was lower as compared to insulin granules in adult β cells (reflecting differences in intracellular insulin content) histotypic differentiation was capable to increase the maturation stage of insulin-positive cells *in vitro*. This would support the idea that a characteristic "biosociology" of pancreatic endocrine cells is necessary for tissue-specific functions (35).

Transplantation of wt and Pax4$^+$ insulin-producing cells was sufficient to preserve normal blood glucose levels in diabetic mice. In contrast to Lumelsky *et al.* (7), we found that wt insulin-producing cells generated by the nestin+ selection protocol also maintained blood glucose. An explanation could be the different location of the implanted cells (kidney capsule and spleen vs. subcutaneous). Indeed, our results are consistent with the results of Soria *et al.* (6) showing blood glucose normalization after intrasplenic transplantation of ES-derived insulin producing cells. Moreover, successful transplantation of purified islets has been shown to be dependent on islet number and implantation site (36).

Tumor formation is an important safety concern when considering transplantation therapies based on ES cells. We observed tumors in the kidney and spleen of some animals transplanted with wt and Pax4$^+$ cells. It is well known that undifferentiated ES cells can form teratomas or teratocarcinomas (see ref. 37). Although Pax4 expression in ES cells resulted in up to 80% insulin-producing cells, remaining undifferentiated cells may still possess oncogenic properties. Lineage selection by using cell-trapping systems (6) and/or fluorescence-activated cell sorting methods similar to those developed for the isolation of β cells (35) have to be further adapted to prevent tumor formation.

In conclusion, our findings present evidence that constitutive Pax4 expression in ES cells, selective differentiation via nestin+ progenitor cells, followed by histotypic maturation, efficiently increases the development of insulin-producing cells.

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Stem cells and pancreatic differentiation in vitro

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Abstract

Cell therapy using pancreatic islets would be a promising therapy to treat diabetes. But, because of the limited supply of human donor islets, other cellular sources have to be considered. Stem cells characterized by extensive proliferation and differentiation capacity may be a valuable source for the in vitro generation of islets. Insulin-producing cells derived from embryonic stem (ES) cells have been shown to reverse experimentally induced diabetes in animal models. However, the oncogenic properties of ES cells are critical in the context of clinical applications and efficient cell-lineage selection systems need to be established. Future studies have to demonstrate whether somatic stem cells residing in adult tissues, such as bone marrow, pancreatic ducts, intestine or liver may provide alternatives to generate functional pancreatic endocrine cells.

Keywords: Embryonic stem cells; Adult stem cells; Pancreatic beta-cells; Development; In vitro differentiation; Insulin; Nestin; C-peptide

1. Introduction

The glucose concentration in the bloodstream of healthy organisms is tightly regulated via hormones of the pancreas, especially insulin. Insulin is produced by endocrine pancreatic beta-cells, the major components of the islets of Langerhans. A dysfunction of beta-cells results in diabetes characterized by an abnormally high blood glucose level and create serious complications including blindness, kidney failure, stroke, heart and vascular diseases.

Whereas type I (insulin-dependent) diabetes is characterized by an auto-immune destruction of insulin-producing cells, type II (non-insulin-dependent) diabetes is characterized by insulin resistance and impaired glucose tolerance, where insulin is inefficiently used or insufficiently produced by beta-cells. The common therapy of diabetes are daily injections of insulin, but this therapy does not solve serious long-term complications, which as a consequence, result in a shortened life expectancy of diabetic patients (Nathan, 1993). Therefore, great efforts have been undertaken to develop alternative strategies.

One of the most promising therapeutic option for the treatment of diabetes is the transplantation of cells producing insulin. Transplantation of

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new insulin-producing beta-cells, in the form of the whole pancreas or isolated islets, in combination with a specific glucocorticoid-free immunosuppression regime (Edmonton protocol, Shapiro et al., 2000), has been shown to ameliorate the disease by eliminating the need for exogenous insulin and normalizing glycosylated haemoglobin levels (for review, see Shapiro et al., 2003; Kaufman and Lowe, 2003; Ricordi, 2003; Titus et al., 2000).

However, freshly isolated islets from at least two immunologically compatible donors are necessary for these transplantations (Shapiro et al., 2000). Because suitable donor tissue is generally limited, a wide-scale application of islet transplantation for diabetic patients is impossible.

An alternative option would be the use of xenogenic tissue, such as porcine islets, but strong immunosuppression and the risk of retroviral infections create barriers for clinical applications (Butler, 1998). Therefore, other cell sources must be considered to generate transplantable insulin-producing cells for a therapy of diabetes.

A promising alternative for the generation of pancreatic islets are stem cells located in embryonic and adult tissues. Stem cells are characterized by self-renewal and the ability to differentiate into various cell types. Propagation and direction of stem cells into the pancreatic lineage in vitro would allow to generate a sufficient amount of transplantable cells.

To date, stem cell researchers are investigating how both pluripotent embryonic and multipotent adult stem cell populations might be used to develop cell therapy strategies.

A key goal of stem cell research is to understand, how differentiation is controlled and how cellular differentiation may be directed. Research on both embryonic and adult stem cells will make a complementary contribution to these objectives. The article gives an overview about the various alternatives to generate pancreatic endocrine cells from embryonic and somatic stem cell sources.

2. Development of pancreatic beta-cells in vivo

The functional unit of endocrine pancreas, the islet of Langerhans, is composed of four cell types: beta-cells secreting insulin; alpha-cells producing glucagons; delta-cells releasing somatostatin; and pancreatic polypeptide-producing (PP) cells. During embryogenesis, the pancreas develops from ventral and dorsal parts of the foregut endoderm. Transcription factors Hnf6, Hlxb9 and Hnf3β are expressed in the region of foregut endoderm committed to the pancreatic fate (Harrison et al., 1999; Jacquemin et al., 2000; Wu et al., 1997). The developing ventral endoderm is surrounded by cardiac mesoderm, whereas the dorsal endoderm is in contact with the notochord. Early pancreas development involves a series of inductive signals from surrounding tissues (for review see Soria, 2001). Signals from notochord repress the expression of Sonic hedgehog (shh), a negative regulator of Pdx1, a key transcription factor of pancreas development (Hebrok et al., 2000). Pdx1 is expressed in the pancreatic endoderm and is essential for its early development and later becomes restricted to a beta-cell fate. In adult animals, Pdx1 regulates insulin gene expression (Soria, 2001). The role of Pdx1 was demonstrated by showing that mutant mice do not develop any pancreatic tissue (Jonsson et al., 1994). Islet-1 (Isl-1), neurogenin-3 (ngn-3), Nkx2.2 and Beta2/NeuroD are other transcription factors involved in the proliferation and specification of early endocrine progenitors (Ahlgren et al., 1997; Naya et al., 1997; Schwitzgebel et al., 2000; Sussel et al., 1998). Two members of the Pax gene family, Pax4 and Pax6, are essential for proper differentiation of endocrine cells (Sosa-Pineda et al., 1997; St. Onge et al., 1997). Pax6 expression is detected throughout pancreas development and is presented in all endocrine cells. In contrast, Pax4 is required for development of cells restricted to the beta- and delta-cell-lineages. Mice lacking Pax4 fail to develop any beta-cells and become diabetic (Sosa-Pineda et al., 1997), while the alpha-cell population is absent in Pax6 mutant mice (St. Onge et al., 1997).

3. Embryonic stem cells

Embryonic stem (ES) cells represent undifferentiated, pluripotent cells with a nearly unlimited self-renewal capacity in vitro, when cultured on mouse feeder cells and/or in the presence of differentiation inhibitory factors, such as cytokines of the interleukine (IL)-6 family (for review see Smith, 2001). Under appropriate conditions, ES cells derived from

undifferentiated cells of mouse (Evans and Kaufman, 1981) and human (Thomson et al., 1998) blastocysts differentiate in vitro into cell types of all primary germ layers (review Wobus, 2001).

The application of the ES cell technology to regenerative cell therapies including the treatment of diabetes, would have important implications. ES cells are considered as a potentially unlimited source of cells suitable for the generation of transplantable insulin-producing cells. ES cells can also be genetically manipulated (Zwaka and Thomson, 2003) to reduce or to avoid graft rejections by the modification of immune responses (see Odorico et al., 2001).

In the first successful experiments to induce pancreatic differentiation, mouse ES cells were transfected with a selective vector (a drug resistance gene under the control of the insulin promoter), followed by cell-lineage selection and maturation. After in vitro differentiation, one transgenic ES cell clone showed regulated insulin release, and after transplantation, normalized glycaemia in streptozotocin-induced diabetic mice (Soria et al., 2000).

When mouse ES cells differentiated spontaneously in the absence of lineage-specific differentiation factors in vitro, only a small cell fraction (0.1%) displayed beta-cell-specific properties, such as staining by zinc chelating dithizone (DTZ) and expression of pancreatic markers at the mRNA level (Shiroi et al., 2002). Under non-selective culture conditions, a small subpopulation of ES cells differentiated into a pancreatic endocrine fate following many facets of early pancreatic development (Kahan et al., 2003). Obviously, the differentiation of ES cells without the application of pancreatic differentiation factors results in a progeny, which does not show functional characteristics of pancreatic beta-cells, such as glucose-dependent insulin release, specific ion channel activity, presence of insulin positive secretory granules or rescue of diabetes in animal models. Therefore, the development of more refined culture techniques proved to be necessary.

Such a strategy for the generation of beta-like cells from ES cells was established by Lumelsky et al. (2001). The method involves a sophisticated five-stage method including the formation of embryoid bodies (EBs), selection and propagation of progenitors expressing the intermediate filament protein nestin and final maturation of cells resulting in the formation of insulin positive cell clusters. These clusters showed regulated insulin release in vitro, but failed to normalize high blood glucose levels in animal models (Lumelsky et al., 2000).

A re-investigation of the differentiation strategy developed by Lumelsky et al. (2001) with mouse and human ES cell lines however showed that the insulin positive cells generated via nestin-positive progenitors concentrated insulin from the culture medium and were characterized by small condensed nuclei and an apoptotic cell status. Although the differentiated cells were insulin positive, the detection of insulin mRNA was inconsistent and weak, and C-peptide, a by-product of insulin synthesis, or secretory granules were not detected (Rajagopal et al., 2003). Two conclusions have been drawn: (i) insulin immuno-staining alone (without demonstration of C-peptide) is not a reliable method to investigate pancreatic differentiation in vitro and only de novo insulin synthesis by C-peptide labelling would demonstrate genuine beta-cell differentiation and (ii) the method based on the selection of nestin-positive cells (via a neural pathway) is irreproducible and therefore not suitable for the generation of pancreatic beta-cells from ES cells.

By the application of a modified protocol based on the differentiation of transgenic ES cells via nestin-positive cells followed by differentiation induction and maturation, we generated functional insulin positive cells (Blyszczuk et al., 2003). We introduced the pancreatic developmental control gene Pax4 into ES cells, which resulted in a four to five fold increased efficiency in the formation of insulin-expressing cells (Blyszczuk et al., 2003). In addition, our protocol included two modifications of the original protocol of Lumelsky et al. (2001): (i) nestin-positive cells were not expanded in the presence of bFGF; and (ii) the nestin-positive cells were treated with pancreatic differentiation factors, such as nicotinamide, insulin and laminin, for 19 instead of 6 days.

By this new approach, we presented evidence for successful pancreatic differentiation from mouse ES cells by: (i) the demonstration of Pax4 mRNA levels in wild-type ES-derived progeny by RT-PCR, (ii) the formation of secretory granules positive for insulin by electron microscopy in Pax4 transgenic ES-derived spheroids, and (iii) a rescue of diabetes in streptozotocin-treated diabetic mice after transplantation of differentiated ES cells (Blyszczuk et al., 2003). Here, we present additional evidence for the capacity

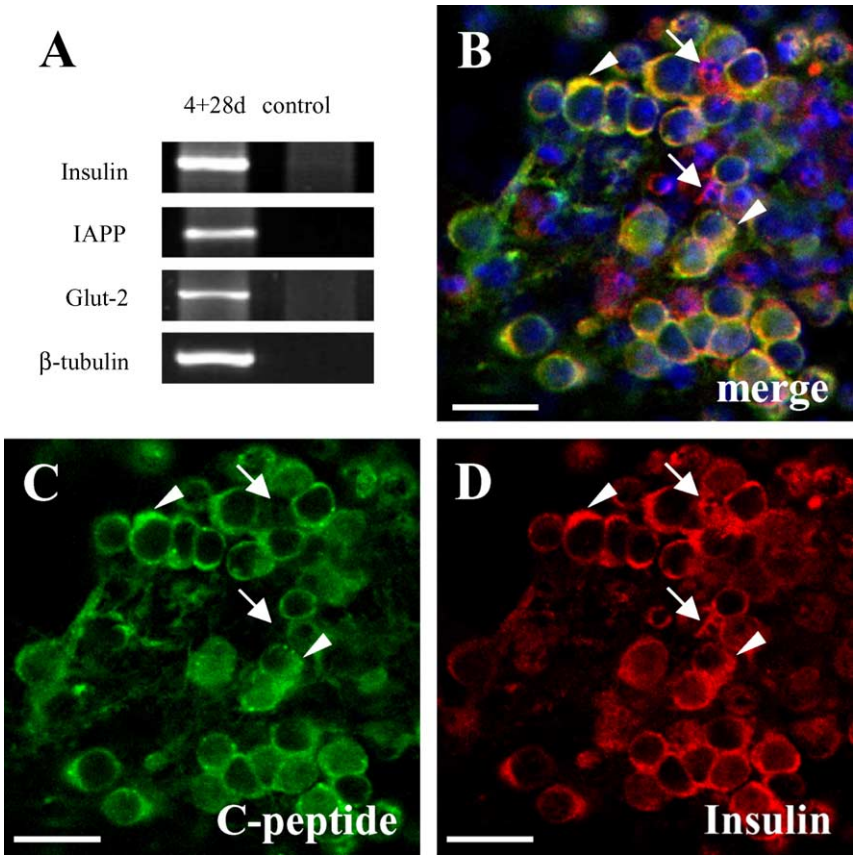

Fig. 1. (A) The presence of insulin, IAPP (islet amyloid polypeptide), Glut-2 (glucose transporter-2) and β-tubulin mRNA transcripts in ES-derived cells analysed by RT-PCR. ES cells were differentiated until stage 4 + 28d (= differentiation as EBs for 4d and for 28d after plating) according to the modified "nestin + selection" protocol (for details, see Blyszczuk et al., 2003). Total RNA was isolated, reverse-transcribed and amplified using specific primers for each gene (for control, samples were amplified in the absence of reverse-transcribed RNA). (B–D) Double immunofluorescence analysis revealed co-labelling (see B) of C-peptide (C) and insulin (D) in islet-like clusters differentiated from ES cells (stage 4 + 28d according to Blyszczuk et al., 2003). Arrowheads point to C-peptide/insulin positive cells (yellow, B), whereas some insulin positive cells (arrows) are negative for C-peptide (red, B). These cells are usually smaller and show fragmented nuclei suggesting apoptosis. Nuclei staining by Hoechst 33342 is shown in blue (B). Bar = 20 μm.

of the differentiated cells by showing that the modified protocol resulted in insulin, IAPP and Glut-2 mRNA levels by RT-PCR (Fig. 1A) and the generation of cells co-expressing insulin and C-peptide (Fig. 1B–D). The data confirm that ES-derived cells are capable of differentiating into pancreatic cells producing de novo insulin. In comparison to the findings published by Rajagopal et al. (2003), we detected a small fraction (10%–15%) of ES-derived insulin positive cells, which do not co-express C-peptide and insulin, but show fragmented nuclei suggesting apoptosis (see Fig. 1). These cells might represent the population that concentrates but not produces insulin (see Rajagopal et al., 2003).

Our data are in agreement with other findings that showed the generation of functional beta-like cells after treatment of ES cells with an inhibitor of phosphoinositide 3-kinase (PI3K) during terminal differentiation. The ES-derived progeny expressed various beta-cell-specific markers (Hori et al., 2002) and, after engraftment into diabetic mice, improved glycaemic control and rescued animal survival.

However, the controversial results of Lumelsky et al. (2001) and Rajagopal et al. (2003) raised questions

for the significance of nestin-expressing cells during pancreatic differentiation in vitro. During development, nestin expression is not detected in pancreatic progenitor cells, but is observed in mesenchymal cells associated with the pancreas, the so-called pancreatic stellate cells (Lardon et al., 2002; Selander and Edlund, 2002). Therefore, it is questionable whether nestin-positive cells, in general, can be regarded as pancreatic progenitors. Because in vivo nestin-positive cells do not represent a homogenous cell population, and nestin is expressed in various progenitor cells of different tissues including the developing central nervous system (Frederiksen and McKay, 1988; Lendahl et al., 1990), skeletal muscle (Sejersen and Lendahl, 1993), heart (Kachinsky et al., 1995), endothelial (Mokry and Nemecek, 1998), mesenchymal pancreatic (Lardon et al., 2002; Selander and Edlund, 2002) and hepatic stellate (Niki et al., 1999) cells, it is more likely that in vitro only a certain sub-population of nestin-positive cells is induced into the pancreatic lineage. Due to minor variations in the cultivation protocol applied by Lumelsky et al. (2001) and Rajagopal et al. (2003), different levels of nestin-expressing sub-populations may be promoted or eliminated during the nestin-selection phase. After differentiation induction, this could result in varying levels of pancreatic (C-peptide-positive) versus neuronal cells.

Until now, the role of nestin-expressing cells in pancreatic differentiation and regeneration is not defined. Further studies have to answer the question whether nestin-positive cells are pancreatic stem cells or represent a progenitor cell type of high plasticity, which in vitro under specific culture conditions may differentiate into the pancreatic lineage.

In first experiments with human ES cells, the spontaneous differentiation via EBs revealed only about 1% of insulin-secreting cells with some characteristics of pancreatic beta-cells, but the cells did not respond to glucose (Assady et al., 2001). Treatment of human ES cells with NGF resulted in the up-regulation of Pdx1 (Schuldiner et al., 2000), a gene known to control insulin transcription and insulin release. So far, the generation of functional beta-like cells from human ES cells by applying the protocol selecting for nestin-positive cells failed (Rajagopal et al., 2003).

Human ES cells routinely have been cultured on mouse feeder cells (Thomson et al., 1998). They are also maintained in the undifferentiated state without feeder cells, but in a feeder cell-conditioned medium (Xu et al., 2001). However, for clinical applications, human ES-derived cells generated without any mouse cells would be necessary in order to avoid potential retroviral contamination. Recently, mouse feeder-free ES cells have been successfully differentiated into glucose-responsive insulin-producing cells (Moritoh et al., 2003).

In conclusion, mouse and human ES cells display the principal capacity to differentiate into pancreatic insulin-producing cell types; however, the functional capacity of beta-like cells has to be significantly improved (see Table 1).

4. Adult stem and progenitor cells

Multipotent adult stem (AS) cells are located in somatic tissues and are known to maintain and regenerate tissues and organ systems. The cellular basis of these properties is the capacity of AS cells to self-renew and to recruit the various cell types of the tissue. AS cells were found not only in tissues characterized by extensive regeneration as bone marrow (De Haan, 2002), skin (Watt, 2001), intestinal epithelium (Potten, 1998) or liver (Theise et al., 1999), but also in many other tissues, such as retina (Tropepe et al., 2000), central nervous system (review Okano, 2002), and skeletal muscle (Seale et al., 2001).

During the last few years, data were published that supported the view that AS cells could serve as an alternative to ES cells also for the generation of pancreatic cells.

With respect to the generation of beta-cells, the most suitable tissue containing pancreatic stem cells is the pancreatic ductal epithelium. The formation of islets is closely associated with pancreatic ductal epithelium, which through sequential steps of differentiation gives rise to all pancreatic endocrine cell types (for review, see Hellerstrom, 1984; Slack, 1995). Although no associated cell markers that define pancreatic progenitors are identified, there are clear evidences for the existence of pancreatic stem/progenitor cells in the ductal epithelium (Bonner-Weir and Sharma, 2002). The cultivation of mouse pancreatic epithelium under appropriate conditions resulted in the differentiation of functional endocrine cells secreting insulin in a glucose-dependent manner and the normalization

Table 1
In vitro generation of insulin-producing beta-like cells from ES and AS cell sources and their functional characteristics

Cell source	Reference	In vitro glucose challenge	Rescue of diabetes in animal models
Mouse ES cells	Soria et al., 2000	+	+
Mouse ES cells	Lumelsky et al., 2001	+	–
Mouse ES cells	Shiroi et al., 2002	–	n.d.
Mouse ES cells	Hori et al., 2002	+	+
Mouse ES cells	Blyszczuk et al., 2003	+	+
Mouse ES cells	Moritoh et al., 2003	+	–
Mouse ES cells	Kahan et al., 2003	n.d.	n.d.
Mouse and human ES cells	Rajagopal et al., 2003	–	n.d.
Human ES cells	Assady et al., 2001	–	n.d.
Mouse pancreatic ductal epithelium	Ramiya et al., 2000	+	+
Human pancreatic ductal epithelium	Bonner-Weir et al., 2000	+	n.d.
Human pancreatic ductal epithelium	Gao et al., 2003	+	n.d.
Nestin-positive cells derived from rat and human pancreatic islets	Zulewski et al., 2001	n.d.	n.d.
Nestin-positive cells derived from human pancreatic islets	Abraham et al., 2002	n.d.	n.d.
Rat hepatic oval cells	Yang et al., 2002	+	+[a]
Mouse intestinal epithelium	Suzuki et al., 2003	+	+
Mouse bone marrow[b]	Ianus et al., 2003	+	n.d.
Mouse bone marrow[c]	Hess et al., 2003	n.d.	+

n.d. = not determined.
[a] Preliminary study.
[b] In vivo differentiation after injection into irradiated mice.
[c] Induction of endogenous pancreatic regeneration.

of high blood glucose levels after implantation into diabetic mice (Ramiya et al., 2000). Similar results were reported for human pancreatic ductal epithelial cells that upon stimulation by specific factors and extracellular matrix (Matrigel) formed three-dimensional glucose-responsive islet-like clusters (Bonner-Weir et al., 2000; Gao et al., 2003). A preliminary study showed that these clusters when transplanted under the kidney capsule of nude mice undergo differentiation into endocrine and exocrine cells (Gao et al., 2003).

As mentioned above, cells expressing the intermediate filament protein nestin are another cell type residing in the pancreas, and have been proposed to represent islet stem or progenitor cells (Hunziker and Stein, 2000; Zulewski et al., 2001), but nestin expression was not associated with epithelial cells throughout pancreatic development (Selander and Edlund, 2002). However, when cultured in vitro, rat and human islet-derived cells expressed nestin, showed a high proliferation capacity and were capable to differentiate into cells not only producing and secreting insulin but also expressing markers of ductal epithelium, exocrine pancreatic cells and hepatocytes (Abraham et al., 2002; Zulewski et al., 2001). Regardless of the controversies about the functional properties of nestin-positive cells in the pancreas, these cells may be considered as a potential cell source for future cell replacement therapies in diabetes (Table 1).

Similar to nestin-positive cells, stem or progenitor cells from adult tissues may possess a higher plasticity and therefore be capable to differentiate into cell types of other lineages (for review, see Blau et al., 2001; Czyz et al., 2003). For example, the successive cultivation of bone marrow-derived mesenchymal cells in vitro resulted in the generation of multipotent adult progenitor cells (MAPCs) with increased developmental potential (Jiang et al., 2002). However, recent reports also showed that the so-called, 'trans-differentiation' capacity of AS cells is rather limited (Kirchhof et al., 2002), could not be repeated (Morshead et al., 2002; Wagers et al., 2002), or may be the result of cell fusions with endogenous cells (Alvarez-Dolado et al., 2003; Terada et al., 2002). Therefore, further detailed studies are needed to solve these questions.

Nevertheless, various AS cells have already been considered as a putative cell source for the in vitro generation of pancreatic beta-cells. During embryogenesis, pancreas and liver develop from the same region of endoderm (Slack, 1995; Zaret, 1996), and signalling molecules from mesoderm specify the cell fate of bipotential precursors (Deutsch et al., 2001). The comparable origin of pancreatic and hepatic cells during embryogenesis and the processes of regeneration in the liver suggest that hepatic stem cells may be one of the most promising non-pancreatic adult stem cell sources. Indeed, liver oval cells, considered as one hepatic stem cell phenotype, were successfully trans-differentiated into glucose-responding insulin-producing cells. First studies showed the ability of these cells to reverse hyperglycaemia in diabetic mice (Yang et al., 2002).

Another tissue related to pancreas development and characterized by extensive self-renewal capacity is the intestinal epithelium of the foregut. After treatment with glucagon-like peptide-1, adult intestinal epithelium developed into glucose-responsive insulin-producing cells, which after transplantation into diabetic mice reversed experimentally induced diabetes (Suzuki et al., 2003).

It is well accepted that bone marrow would be an ideal source for the generation of pancreatic cells. Experiments showed that bone marrow-derived cells may have the capacity to differentiate into non-haematopoietic cell types when transplanted into irradiated hosts (review Blau et al., 2001), but only recent studies presented evidence for a pancreas-specific development of bone marrow-derived cells. A Cre-LoxP system allowed the identification and purification of bone marrow-derived insulin-expressing cells and these cells showed glucose-dependent insulin-release (Ianus et al., 2003). Another recent study showed that transplantation of adult bone marrow-derived cells reduces hyperglycaemia in diabetic mice by initiating endogenous pancreatic tissue regeneration. Engraftment of bone marrow-derived cells to ductal and islet structures was accompanied by rapid proliferation of recipient pancreatic cells and neogenesis of insulin-producing cells of recipient origin. This strategy may represent a previously unrecognised means by which bone marrow-derived cells can contribute to tissue restoration (Hess et al., 2003, see Table 1).

The use of adult stem cells for the treatment of diabetes would include, in principle, the isolation of stem cells, in vitro proliferation and differentiation into pancreatic cells and implantation into a diabetes patient. The transplantation of autologous cells would exclude immuno-suppression to inhibit graft rejection. However, it is still unclear whether such therapy will be applicable for patients with type I diabetes because in vitro generated beta-cells may be also destroyed by auto-immune reactions as native beta-cells (see Peck et al., 2002).

5. Tumour formation and cell-lineage selection systems

Mouse and human ES cells possesses an enormous differentiation potential; however, they also display many features of cancer cells, such as unlimited proliferation capacity, clonal growth and lack of contact inhibition (Burdon et al., 1999), that result in teratoma or teratocarcinoma formation when transplanted into syngeneic animals (Stevens, 1983; Thomson et al., 1998; Wobus et al., 1984). The present studies showed that the oncogenic properties of ES cells are not eliminated during pancreatic differentiation in vitro. Although the ES-derived cells represent differentiated pancreatic cells that are able to rescue diabetes, the remaining undifferentiated cell fraction may be a cell population with tumorigenic potential (Blyszczuk et al., 2003; Hori et al., 2002). The data showed that cultivation strategies based on promoting pancreatic differentiation by growth and extracellular matrix factors alone did not avoid the cellular heterogeneity and tumorigenicity.

Therefore, strategies for the active elimination of tumorigenic cells, or more specifically, for a selective isolation of differentiated phenotypes are needed. Purification strategies by the introduction of drug resistance genes under the control of lineage-specific promoters followed by drug selection of ES cell clones or of ES-derived cells after specific differentiation induction have been reported for neuronal (Li et al., 1998), cardiac (Klug et al., 1996) and pancreatic cells (Soria et al., 2000). In alternative selection systems, the drug resistance gene is replaced by the gene encoding the enhanced green fluorescent protein (EGFP), or enhanced cyan fluorescent protein (ECFP) and instead of drug selection, fluorescence activated cell sorting (FACS) is used for cell isolation. These procedures have already been applied for the isolation of several

cells, including early cardiac cells (Kolossov et al., 1998) and ventricular cardiomyocytes (Meyer et al., 2000; Müller et al., 2000).

An advantage of the lineage selection strategy is the elimination not only of tumorigenic cells but also of other non-pancreatic cells. The use of the insulin promoter and selection of ES cell clones expressing insulin as described by Soria et al. (2000), however, did not guarantee that cells undergo full differentiation and lose their oncogenic potential. One reason might be that insulin is expressed at low levels also in undifferentiated ES cells.

Therefore, selection strategies using lineage-specific promoters, which are exclusively active in non-tumorigenic cells and additional techniques will be necessary to avoid tumour formation after transplantation.

Labelling of specific cell surface markers followed by FACS selection may be a supplementary method to isolate the cell types of interest. Antibodies against glycolipids or other cell surface markers of pancreatic beta-cells, such as A2B5 (Eisenbarth et al., 1982), 3G5 (Powers et al., 1984), R2D6 (Alejandro et al., 1984) or IC2 (Brogren et al., 1986) may be considered for the isolation of pancreatic cells from ES cell cultures. It has to be investigated, whether the composition of cell surface markers of ES-derived pancreatic cells differs from those expressed in islets, or the presented antibodies bind also to other cell types, for example neuronal cells. Further studies are needed to answer these questions.

The engineering of pancreatic islets in vitro requires further maturation of the cell clusters. The specific isolation of pancreatic cells from the culture would allow to select cells expressing beta-cell-specific signals from non-pancreatic cells, especially in ES-derived cultures. It has been demonstrated that cell-to-cell interactions and the characteristic "bio-sociology" of pancreatic endocrine cells are necessary for tissue-specific function (Pipeleers, 1987). This could be achieved by a histotypic culture system (Blyszczuk et al., 2003) that is additionally supported by micro-vascularization. Finally, the maturation process could be enhanced by delivery of specific pancreatic transcription factors or developmental control genes in a 'gain-of-function' approach. To avoid the genetic modification of cells used for transplantation, also protein transduction techniques may be a useful alternative (Elliott and O'Hare, 1997; Schwarze et al., 1999).

Although we have to realize that we are far away from applicable cell therapy strategies for the treatment of diabetes, the new technical achievements of cell and molecular biology will positively influence stem cell research and hopefully, in the future, will result in the generation of functional islets for clinical applications.

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Original Article

Embryonic stem cells differentiate into insulin-producing cells without selection of nestin-expressing cells

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ABSTRACT We present a new strategy for the differentiation of embryonic stem (ES) cells into insulin-producing cells via a multi-step process without selection and induction of nestin-positive cells. During ES cell differentiation, transcript levels of genes characteristic of early and mature beta cells including Pdx1, Pax4, insulin and islet amyloid pancreatic peptide are up regulated. Islet-like clusters are characterized by expression of C-peptide, insulin and partially cytokeratin 19 as well as by ion channel activity similar to that found in embryonic beta cells. Cells of islet-like clusters show glucose-dependent insulin release at terminal stage. At an intermediate stage, nestin is partially co-expressed with C-peptide and cytokeratin 19, whereas islet-like clusters at the terminal stage are nestin-negative. We conclude that expression of nestin and cytokeratin 19 is a normal property of ES cells preceding differentiation into C-peptide/insulin-producing cells without any selection for nestin-positive phenotypes.

KEY WORDS: *mouse embryonic stem cell, differentiation, C-peptide, insulin-producing cell, nestin*

Introduction

A potential source of transplantable cells for the treatment of diabetes are embryonic stem (ES) cells, which have an almost unlimited proliferation capacity, while retaining the potential to differentiate *in vitro* into cells of all three primary germ layers [for review, see (Czyz *et al.*, 2003)]. Insulin-expressing cells have been generated from mouse (Soria *et al.*, 2000; Leon-Quinto et al., 2004) and human (Assady *et al.*, 2001) ES cells. Insulin-positive cells were also derived from mouse ES cells via selection of progenitor cells expressing the intermediate filament (IF) protein nestin (Lumelsky *et al.*, 2001). However, it was recently suggested that insulin-positive cells derived from ES cells absorbed and concentrated the hormone from the medium rather than producing insulin (Rajagopal *et al.*, 2003; Hansson *et al.*, 2004); whereas other reports described ES cell differentiation into transplantable insulin-producing cells *via* nestin-positive cells by treatment with a phosphoinositide 3-kinase inhibitor (Hori *et al.*, 2002) and by *Pax4* transgene expression (Blyszczuk *et al.*, 2003), respectively.

In vivo, nestin expression during pancreas development was only detected in mesenchymal and not in pancreatic epithelial cells (Selander and Edlund, 2002). Whereas cytokeratin 19 (CK19) was found to be expressed in the developing pancreas and in pancreatic duct cells, where potential pancreatic stem cells reside (Bouwens, 1998; Bonner-Weir *et al.*, 2000; Brembeck *et al.*, 2001), nestin was not identified as a specific marker of beta-cell precursors (Humphrey *et al.*, 2003). However, after cultivation of pancreatic islets *in vitro*, nestin-positive cells were detected (Zulewski *et al.*, 2001). The controversial findings substantiated our studies to characterize nestin and CK 19 expression during differentiation of ES cells into C-peptide/insulin-positive cells and To this aim, we established a new protocol for the differentiation of ES cells into the pancreatic lineage without pre-selection of nestin-positive cells, determined the abundance of C-peptide-, CK19- and nestin-positive cells and analyzed the functional capacity of differentiated cells. We found a co-expression of nestin with C-peptide and CK19 at intermediate stage, but not in islet-like clusters. Our data let us

Abbreviations used in this paper: EB, embryoid body; ES, embryonic stem; bFGF, basic fibroblast growth factor; CK, cytokeratin; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme linked immunosorbent assay; FCS, fetal calf serum; IAPP, islet amyloid polypeptide; IF, intermediate filament; IMDM, Iscove's modified Dulbecco's medium; ITSFn, insulin, transferrin, selenium, fibronectin; PBS, phosphate buffered saline; PFA, paraformaldehyde; RT-PCR, reverse transcriptase-polymerase chain reaction; SEM, standard error of the mean; STZ, streptozotocin; wt, wild type.

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to conclude that nestin and CK 19 are involved in pancreatic differentiation *in vitro* and that cells differentiating along these pathways acquire some characteristics of pancreatic beta-like cells.

Results

Transcript levels of pancreatic genes are upregulated in ES-derived cells differentiating into insulin-producing cells without selecting nestin-positive cells

Our new differentiation protocol for the generation of ES-derived insulin-producing cells (Fig. 1C) includes the spontaneous generation of multi-lineage progenitor cells and their differentiation induction into islet-like clusters. No selection for nestin-positive cells is performed at any time of ES cell differentiation. In brief, ES cells were cultured in 'hanging drops' and in suspension for 5 days to form embryoid bodies (EBs) followed by 9 days culture after EB plating (= 5+9d) for differentiation into cells representing derivatives of all three primary germ layers [for details see (Rohwedel *et al.*, 2001; Wobus *et al.*, 2002; Blyszczuk *et al.*, 2003)]. At this stage, cells were re-plated onto poly-L-ornithine/laminin-coated culture dishes [see (Rolletschek *et al.*, 2001)] into a 'pancreatic differentiation medium' (= N2 medium + nicotinamide, NA, see Fig. 1C) and cultured until stage 5+28d. Cells were analyzed for pancreatic differentiation markers at stages 5+9d, 5+16d and 5+28d. In the present study and in previous reports (Lumelsky *et al.*, 2001; Hori *et al.*, 2002; Moritoh *et al.*, 2003; Rajagopal *et al.*, 2003; Blyszczuk *et al.*, 2003), the same 'pancreatic differentiation medium' was used to induce pancreatic differentiation. However, in contrast to Lumelsky *et al.* (2001), our new protocol avoids the enrichment of nestin-positive cells in ITSFn medium [see Lee *et al.* (2000), used also in our former study (Blyszczuk *et al.*, 2003); see Fig. 1B] and the proliferation induction of nestin-positive cells by bFGF [according to Lumelsky *et al.* (2001); compare Fig. 1 A,C]. Therefore, our new protocol allows the characterization of cells during pancreatic differentiation of ES cells without enrichment of specific subpopulations that may affect commitment and differentiation into the pancreatic lineage.

Employing RT-PCR and immunofluorescence techniques we show that by applying our new protocol ES cells differentiate into the pancreatic lineage. Upon induction of pancreatic differentiation at day 5+9, development into pancreatic endocrine cells is demonstrated by the up-regulation of Pdx1, Pax4, insulin and IAPP mRNA levels (Fig. 2A) resulting in the formation of self-assembling islet-like clusters, which are labelled by insulin and C-peptide following differentiation (Fig. 2B).

Pdx1 transcripts were detected at low level at day 5+9, showed no Pdx1 signals at the intermediate stage (5+16d), but increased significantly at the terminal stage (5+28d). Lack or low Pdx1 expression at early stages *in vitro* confirms also recent findings which show that Pdx1 is not required for the induction of pancreatic differentiation from ES cells *in vitro* (; although it plays a key role in early pancreas development

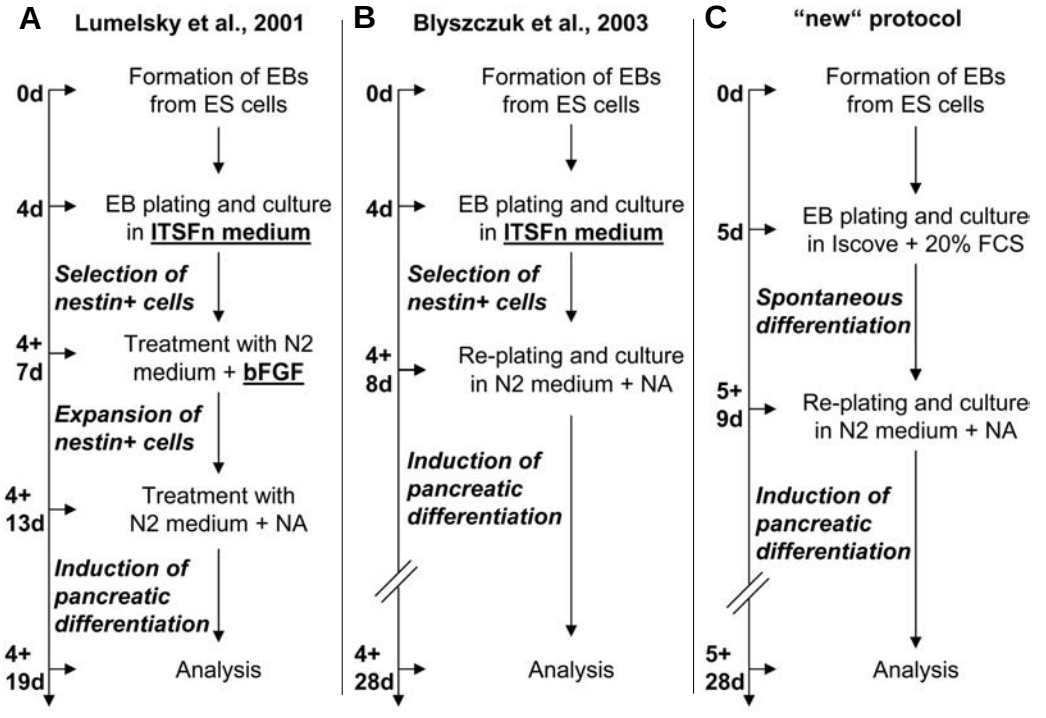

Fig. 1. Schematic representation of protocols used to generate functional islet-like clusters from ES cells *in vitro*. (A-C) *The protocols developed by* **(A)** *Lumelsky* et al. *(2001),* **(B)** *that employed in our former study [Blyszczuk* et al. *(2003)] and* **(C)** *that of the present study ("new" protocol), differ in the generation of progenitor cells, but all protocols use the same pancreatic differentiation medium. All protocols generate progenitor cells from ES cells via EB formation, but protocols (A) and (B) enrich nestin-positive cells in ITSFn supplemented medium, whereas according to the «new» protocol (C), EB-derived cells differentiate spontaneously. A further expansion of nestin-positive cells by bFGF is performed only in protocol (A), whereas in methods (B) and (C) the selective expansion of nestin-positive cells is omitted. According to our «new» protocol (C), a progenitor population representing cells of all three primary lineages is generated by spontaneous differentiation of ES cells followed by the induction of pancreatic differentiation.*

(Jonsson *et al.*, 1994) as well as in terminal differentiation and functionality of pancreatic cells (Ahlgren *et al.*, 1998).

Another evidence for the activation of a pancreatic differentiation program in ES cells by following our protocol, is the up-regulation of insulin and IAPP, a peptide almost exclusively expressed in beta-cells and co-secreted with insulin (Fehmann *et al.*, 1990).

Pax4 transcripts were up regulated upon induction of pancreatic differentiation at 5+16d and 5+28d. *In vivo*, Pax4 is essential for proper beta-cell development (Sosa-Pineda *et al.*, 1997) and its expression is restricted to the endocrine progenitor cell fate (Dohrmann *et al.*, 2000). We have recently reported that constitutive expression of Pax4 in ES cells significantly promotes pancreatic differentiation (Blyszczuk *et al.*, 2003).

To test whether ES-derived insulin-positive cells produce or only absorbed insulin from the medium [see (Rajagopal *et al.*, 2003)], we analysed co-expression of insulin and C-peptide. The formation of C-peptide (part of proinsulin when converted into insulin) demonstrates *de novo* insulin production and excludes those cells that only concentrate insulin from the medium. The number of C-peptide-labelled cells increased after induction of pancreatic differentiation up to about 3 fold at terminal stage (5+28d, Table 1). A high fraction of insulin-positive cells (about 85%) co-expressed C-peptide (see arrowheads in Fig. 2B) and were organized in islet-like clusters at terminal stage of 5+28d (Fig. 2B), whereas only a low fraction (about 10- 15%) of cells were labeled by insulin, but not by C-peptide (see arrows in Fig. 2B). These cells were eventually characterized by small, condensed nuclei suggesting an apoptotic pathway as described (Rajagopal *et al.*, 2003). In contrast to Rajagopal *et al.* (2003), we showed co-expression of insulin and C-peptide in most of the ES-derived cells indicating *de novo* insulin production and, as demonstrated in Fig. 2A, high insulin mRNA levels were determined at terminal stages.

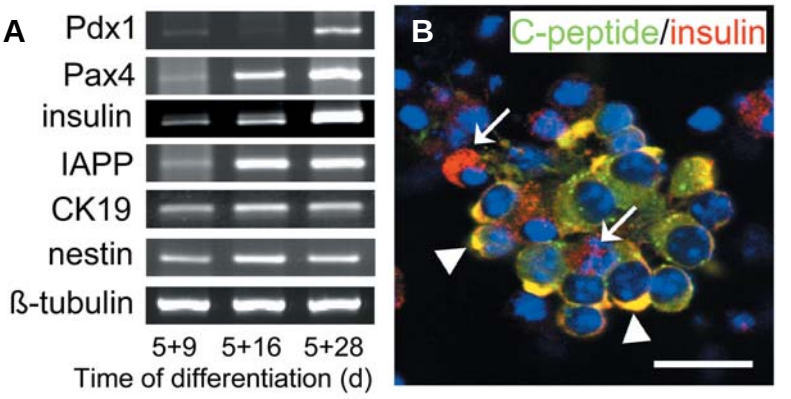

Fig. 2. Transcript levels and an islet-like cluster after pancreatic differentiation of ES cells. (A) *mRNA levels of* Pdx1, Pax4, insulin, IAPP, cytokeratin (CK)19, nestin *and* β-tubulin *(housekeeping) genes of ES-derived cells during* in vitro *differentiation are shown at stages 5+9d, 5+16d and 5+28d.* **(B)** *Immunofluorescence of C-peptide and insulin co-expressing cells in an islet-like cluster at terminal stage of differentiation (5+28d). Arrowheads indicate insulin/C-peptide co-expressing cells, whereas arrows indicate insulin-positive and C-peptide-negative cells with small and condensed nuclei suggesting apoptosis. Nuclei are labelled by Hoechst 33342. Bar, 20 μm.*

Nestin is coexpressed in C-peptide-positive cells after induction of pancreatic differentiation

To answer the question, whether nestin is involved in the generation of insulin-producing cells during ES cell differentiation, we analysed transcript abundance and co-localization of nestin and C-peptide by immunostaining at stages 5+9d, 5+16d and 5+28d. Nestin mRNA levels were detected at all stages (see Fig. 2A) and nestin-positive cells were present at constant level during differentiation between day 5+9 and 5+28 (Table 1).

A high co-expression of nestin and C-peptide was found exclusively at stage 5+16d (Fig. 3G, Tables 2A and 2B). Continued differentiation up to 5+28d resulted in C-peptide expressing islet-like clusters negative for nestin (Fig. 3K). Our findings demonstrate that nestin is transiently involved in ES-derived pancreatic differentiation.

To determine, if nestin-positive cells co-express desmin [another IF protein detected in mesenchymal cells and in pancreatic stellate cells, see (Lardon *et al.*, 2002)] and whether such cells are involved in pancreatic differentiation of ES cells, we performed double immunostaining with desmin- and nestin-specific antibodies. Partial co-expression of nestin and desmin was detected at

TABLE 1

NUMBER OF NESTIN-, C-PEPTIDE-, CYTOKERATIN (CK) 19, CARBONIC ANHYDRASE II- (CARB. ANH. II), DESMIN- AND ISLET-1 (ISL-1)-IMMUNOREACTIVE CELLS AS A PERCENTAGE OF HOECHST 33342-LABELLED CELLS DURING PANCREATIC DIFFERENTIATION OF R1 ES CELLS

Stage	Number of positive cells						
	Nestin (%)	C-peptide (%)	CK19 (%) MeOH:Ac	CK19 (%) 4% PFA	Carb. anh. II (%)	Desmin (%)	Isl-1 (%)
5+9d	23.7 ± 5.3	9.9 ± 1.7	8 ± 2.2	< 1.0	n.d.	32.5 ± 5.9	3.1 ± 1.9
5+16d	21.7 ± 4.8	32.4 ± 3.3	< 1.0	31.6 ± 6.2	32.2 ± 4.5	20.1 ± 3.7	15.4 ± 7.1
5+28d	21.4 ± 4.3	26.6 ± 7.7	< 1.0	21.3 ± 1.3	26.6'± 8.0	n.d.	8.7 ± 2.9

Cells of each immunopositive fractions partially overlap. Differentiation stage 5+9d represents spontaneous differentiation, whereas stages 5+16d and 5+28d represent 7 and 19 days, respectively, after induction of pancreatic differentiation. Immunostaining for CK19 was performed separately after fixation with methanol-acetone (MeOH:Ac) and 4 % paraformaldehyde (PFA). Each value represents mean ± SEM, n.d. = not determined, n ≥ 3 experiments.

TABLE 2

DOUBLE IMMUNOFLUORESCENCE ANALYSIS OF ES-DERIVED INTERMEDIATE CELL STAGES REPRESENTING NESTIN (A) AND C-PEPTIDE (B) CELL POPULATIONS

(A)

Stage	Nestin (%)	C-peptide (%)	Isl-1 (%)	Desmin (%)
5+9d	100	34.1 ± 14.5	1.0 ± 0.9	46.1 ± 10.3
5+16d	100	83.9 ± 7.1	60.4 ± 13.7	15.0 ± 6.2
5+28d	100	41.0 ± 6.7	8.5 ± 3.6	n.d.

Number (in %) of C-peptide-, Islet-1 (Isl-1)- and desmin-labelled cells determined within the fraction of nestin-positive cells (nestin-positive cells were set to 100%). Each value represents mean ± SEM, n.d. = not determined, n ≥ 3 experiments.

(B)

Stage	C-peptide (%)	Nestin (%)	Carb. anh. II (%)	CK19 (%)
5+16d	100	82.2 ± 6.9	99.8 ± 0.3	97.6 ± 0.8
5+28d	100	30.7 ± 6.7	99.7 ± 0.2	73.0 ± 1.4

Number (in %) of nestin-, carbonic anhydrase II (carb. anh. II)- and cytokeratin (CK) 19-labeled cells as a percentage of the fraction of C-peptide-positive cells (C-peptide-positive cells were set to 100%). Each value represents mean ± SEM, n ≥ 3 experiments.

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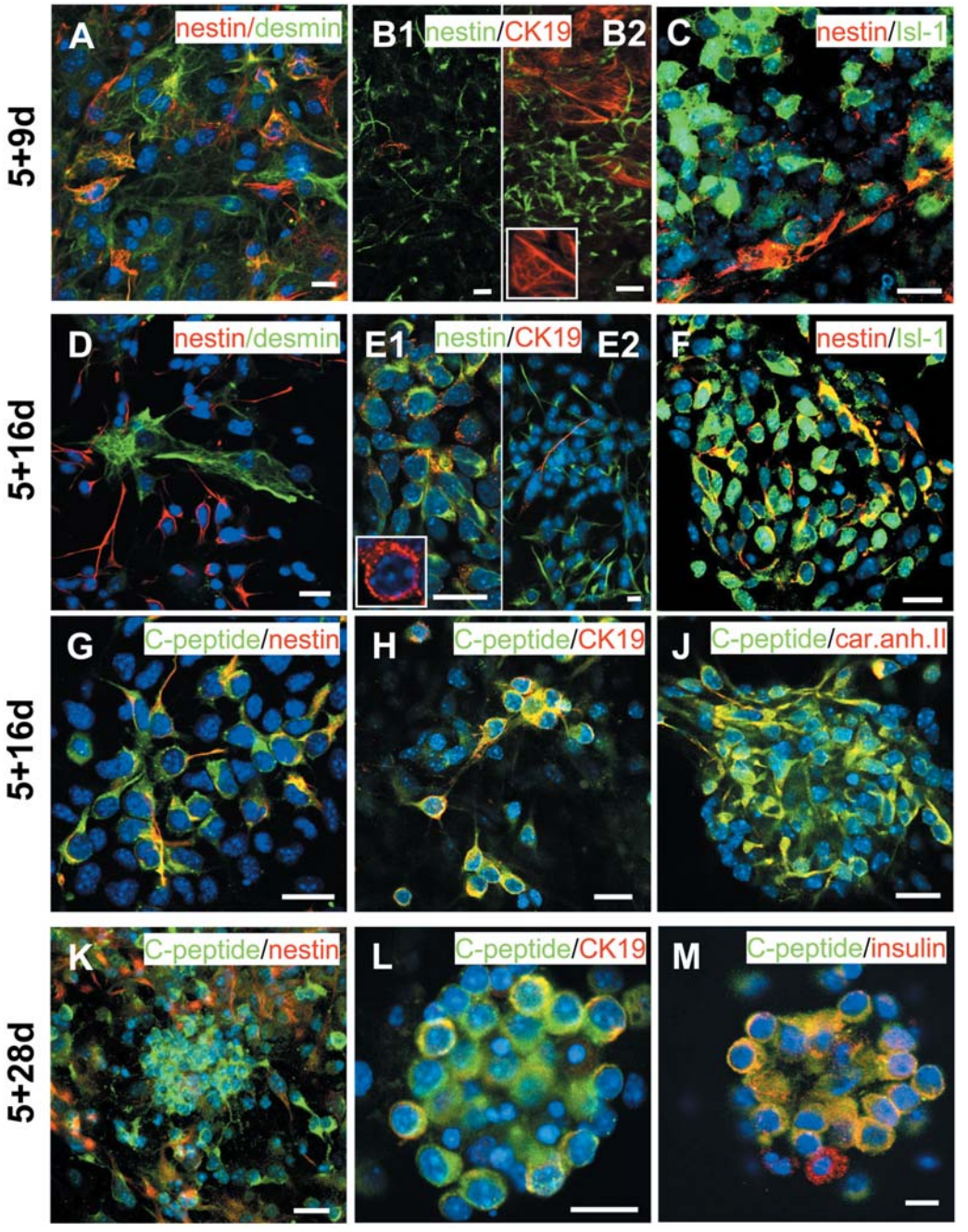

Fig. 3. Double-immunofluorescence analysis of ES-derived cells at intermediate and terminal stages following pancreatic differentiation. **(A-F)** *Analysis of nestin-labelled cells for co-expression with desmin* **(A,D)**, *cytokeratin (CK) 19 [fixed with paraformaldehyde,* **(B1, E1)** *and methanol: acetone* **(B2, E2)***], Isl-1* **(C,F)**, *both fixed with paraformaldehyde, at early (5+9d, A-C) and intermediate (5+16d, D-F) stages of differentiation. Note that not all cells show nuclear Isl-1 labelling in (3F).* **(G-M)** *Immunofluorescence analysis of C-peptide-labelled cells for co-expression with nestin* **(G,K)**, *CK19 [fixed with paraformaldehyde,* **(H,L)***] carbonic anhydrase II (carb. anh. II,* **(J)***) and insulin* **(M)** *at intermediate (5+16d, G-J) and terminal (5+28d, K-M) stages of differentiation. Quantitative analysis of the immunofluorescence signals is presented in Tables 1 and 2, respectively. Nuclei are labelled by Hoechst 33342. Bar, 20 μm.*

stage 5+9d (Fig. 3A). Whereas, upon induction of pancreatic differentiation only a low cell fraction co-expressed nestin and desmin (Table 2A), while the remaining nestin- and desmin-positive cells showed clear differences in the morphology (see Fig. 3D). Moreover, the total number of desmin-positive cells decreased after induction of pancreatic differentiation (see Table 1). These findings suggest that desmin is not involved in the terminal differentiation of pancreatic cells.

Cytokeratin 19 is expressed during ES-derived pancreatic differentiation, but is not organized into filaments

Because cytokeratin 19 (CK19) is expressed in developing pancreatic epithelium, where the definitive pancreatic progenitor reside and in adult pancreatic duct cells (Brembeck *et al.*, 2001), where potential pancreatic stem cells may be located (Bouwens, 1998; Bonner-Weir *et al.*, 2000; Ramiya *et al.*, 2000), next we asked whether CK19 is involved in the generation of C-peptide/insulin-positive cells during ES cell differentiation.

CK19 transcripts were detected at all stages of pancreatic differentiation (see Fig. 2A) suggesting the involvement of CK19 during ES cell differentiation. However, immunofluorescence analysis demonstrated specific differences in the organization of CK19-positive intermediate filaments by applying different fixation methods. Conventional fixation with methanol/ acetone was suitable for detection of intermediate filaments (Fig. 3 B2, E2) and we found a low fraction of CK19-positive cells at stage 5+9d, whereas after

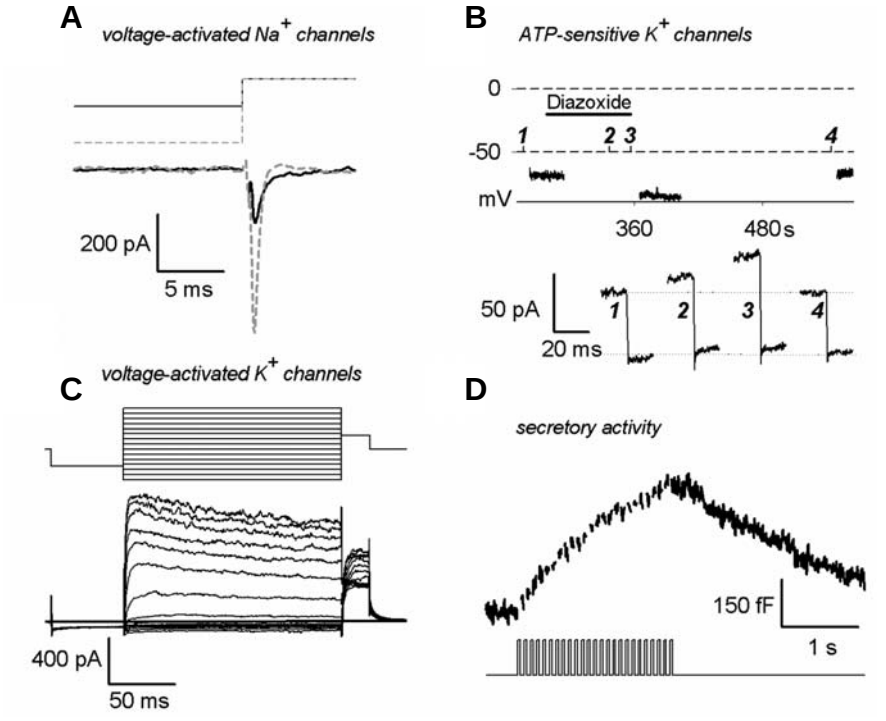

Fig. 4. Results of whole-cell patch-clamp analysis of wt and Pax4+ ES-derived cells from islet-like clusters at stage 5+30d. **(A)** *Inactivation of voltage-activated Na+ currents at sub-physiological membrane potentials. Depolarisation to -20 mV from -75 mV and -150 mV generated inward currents of 210 and 640 pA, respectively.* **(B)** *Voltage-activated K+ currents induced by a family of depolarising pulses in steps of 15 mV.* **(C)** *Current-clamp recording of a cell treated with diazoxide (100 υM). At points indicated by numbers, K+ conductance was measured using square hyper-polarization from -60 mV to -110 mV. Cell hyper-polarization during diazoxide superfusion is associated with significant increase in membrane conductance.* **(D)** *Typical increase in membrane capacitance triggered by a train of depolarising pulses from -80 mV to +10 mV indicates Ca^{2+}-dependent exocytosis.*

pancreatic differentiation induction (5+16d), almost no CK19-positive cells were found (Table 1). In addition, the cells showed no co-expression of CK19 and nestin at early (5+9d) and intermediate (5+16d) stages (Fig. 3 B2, E2). After fixation with 4% paraformaldehyde, CK19-labelled dot-like structures were detected in the cytoplasm of a few cells at stage 5+9d (Fig. 3B1), however upon induction of pancreatic differentiation (stage 5+16d), CK19 labeling was found in 31.6% cells (Table 1) and was highly co-expressed with nestin (Fig. 3E1, Table 2A). After continued differentiation (stage 5+28d), the number of CK19-positive cells was reduced (21.3%, Table 1). Similar dot-like structures were described for another IF protein, vimentin, after fixation with paraformaldehyde (Prahlad *et al.*, 1998). We conclude that the different fixation properties reflect conformational changes (intermediate filaments versus dot-like structures) of the IF proteins during differentiation.

Next, we determined, whether CK19-positive cells are present in the fraction of C-peptide-positive cells that are involved in the formation of islet-like clusters *in vitro*. Double immunofluorescence analysis showed that 97.6% (5+16d) and 73% (5+28d) of C-peptide positive cells co-expressed CK19 (Fig. 3 H,L, Tab. 2B). These data suggest that CK19 protein is abundant during pancreatic differentiation, but the protein is not organized in intermediate filaments**.** The decrease of CK19-labeling in C-peptide-positive cells at terminal stage may indicate a certain maturation of ES-derived cells during differentiation. This would be in agreement with the finding that mature beta cells *in vivo* are negative for CK19 (Bouwens *et al.*, 1997).

Islet-1 is expressed in ES-derived progenitor cells

The data presented so far have shown that nestin and CK19 are partially and transiently co-expressed in C-peptide/insulin-producing cells. Next, we asked whether Isl-1, a LIM homeodomain protein that controls cell-fate decisions required for the differentia-

A

Cell line	Intracellular insulin (ng/mg protein)	Released insulin (ng/mg protein) at 5.5 mM glucose	Released insulin (ng/mg protein) at 27.7 mM glucose
wt	38.1 ± 4.7	6.1 ± 1.3	8.6 ± 1.0
Pax4+	168.0 ± 28.5	17.3 ± 2.9	27.0 ± 3.2

B

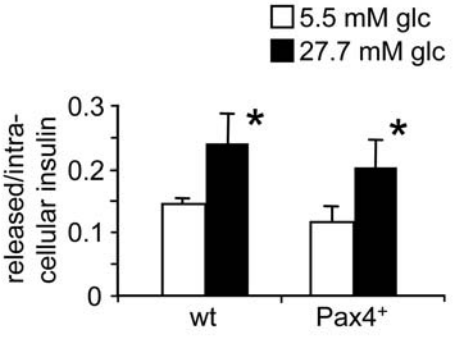

Fig. 5. Determination of insulin levels in ES-derived cells after pancreatic induction by ELISA. **(A)** *Levels of intracellular and released insulin in response to 5.5 mM and 27.7 mM glucose, respectively, determined after 15 min of static incubation of wt and Pax4+ ES-derived cells at stage 5+28d.* **(B)** *Representation of glucose-dependent insulin release by the ratio of secreted and intracellular insulin levels. Each value represents mean ± SEM. Statistical significance was tested by the Student t test: *, P<0.05.*

tion of islet cells (Ahlgren *et al.*, 1997) is expressed during ES cell differentiation. We found only a low fraction of Isl-1-positive cells at the early differentiation stage of 5+9d and almost no cells co-expressed Isl-1 and nestin (Fig. 3C, Tables 1 and 2A). However, after induction of pancreatic differentiation, 60.4% of nestin-positive cells co-expressed Isl-1 and only occasionally cells positive for Isl-1 and negative for nestin were found (Table 2A). Continued differentiation resulted in a decreased number of Isl-1-positive cells at stage 5+28d (Table 1). These findings suggest that Isl-1 is transiently expressed in the nestin-positive subpopulation of committed pancreatic cells expressing nestin and C-peptide (at stage 5+16d). The data are in agreement with recent findings, which showed that Isl-1 expression is restricted to a specific subpopulation of pancreatic stem cells during pancreas development (Chiang and Melton, 2003). Our immunofluorescence analyses suggest that Isl-1 is activated in nestin-positive cells committed to the pancreatic lineage, because a remarkable number of cells co-expressing Isl-1 and nestin were detected at day 5+16d (see Fig. 3F, Table 2A).

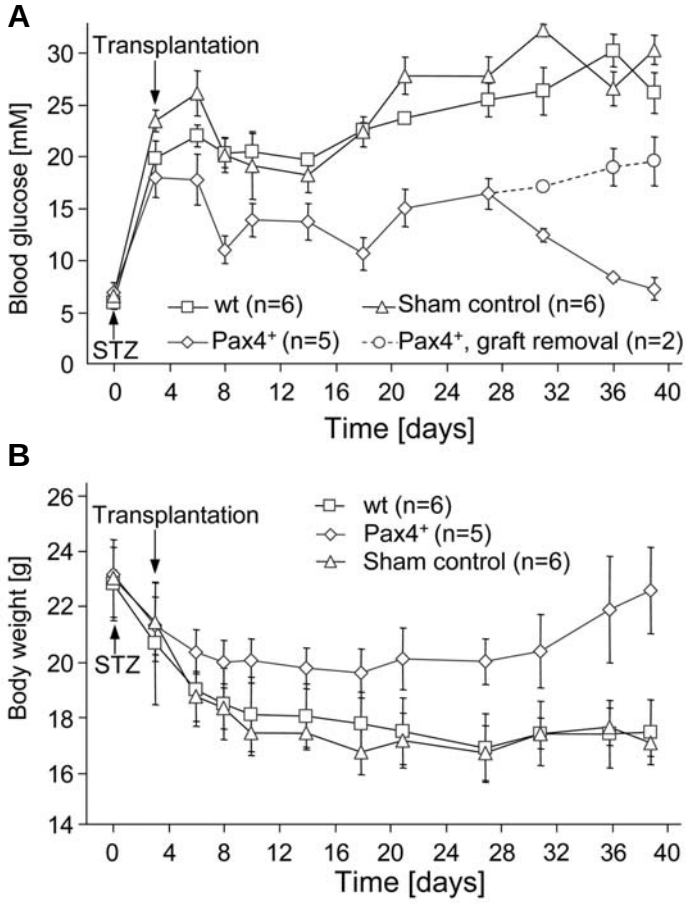

Fig. 6. Transplantation of wt and Pax4+ ES-derived cells at stage 5+28d into STZ-treated diabetic mice and body weight of animals after transplantation. **(A)** *Non-fasted blood glucose levels decreased in animals transplanted with Pax4+ insulin-producing cells as compared to animals receiving wt cells or sham transplants. In two mice transplanted by Pax4+ cells, unilateral nephrectomy was performed 26 days after transplantation to remove substantial parts of the graft.* **(B)** *Transplantation of Pax4+ ES-derived cells ameliorates weight loss in diabetic mice in contrast to wt cells and sham transplants.*

***Carbonic anhydrase II is involved in pancreatic differentiation* in vitro**

Another protein expressed in pancreatic duct cells is carbonic anhydrase II (Githens *et al.*, 1992). We investigated the abundance of carbonic anhydrase II in C-peptide-positive ES-derived cells and found that upon induction of pancreatic differentiation nearly all C-peptide-positive cells co-expressed carbonic anhydrase II (Fig. 3J, Table 3). *In vivo*, mature pancreatic beta-cells express carbonic anhydrase V instead of carbonic anhydrase II (Parkkila *et al.*, 1998) suggesting that *in vitro* generated ES-derived cells represent an immature pancreatic cell population, rather than definitive mature pancreatic beta-cells.

Insulin-producing cells functionally express beta-cell-specific properties

Next, we investigated functional properties of ES-derived wt cells in comparison to ES cells constitutively expressing Pax4 (Pax4+). We have reported that Pax4+ cells promote pancreatic differentiation (Blyszczuk *et al.*, 2003) and confirmed this by applying our new differentiation protocol. Pax4+ cells formed C-peptide-positive islet-like clusters similar as described for wt cells, but showed a higher level of C-peptide-labelled cells (not shown). The functional status of both cell lines was analysed by electrophysiological studies using the patch-clamp technique on cells found within islet-like clusters and by ELISA. Electrophysiological measurements showed that ES-derived pancreatic cells were excitable and revealed characteristic properties of beta-cells with respect to voltage-activated Na+ and K+ currents (Fig. 4 A,B, Table 3). In some Pax4+ cells, KATP channels were responsive to the specific channel opener diazoxide (Fig. 4C, Table 3) and secretory activity was induced via activation of voltage-activated Ca2+ channels (Fig. 4D, Table 3). The characteristics of voltage-activated Na+ channel inactivation, voltage-activated K+ channel properties and the Ca2+-dependent secretory activity in ES-derived pancreatic cells (Table 3) suggest a similarity of ES-derived pancreatic cells to embryonic beta-cells. The presence of cells responsive to KATP channel agonists and glucose-induced secretory activity of Pax4+ ES-derived cells represent a developmentally advanced status in comparison to wt cells.

To further determine the functional status of insulin-producing cells, we analyzed glucose-dependent insulin release by ELISA. We found that both cell lines released higher insulin levels in response to 27.7 mM glucose compared to 5.5 mM glucose (Fig. 5A). Pax4+ cells showed higher insulin levels, but the ratio of released/intracellular insulin level was similar for wt and Pax4+ cells (Fig. 5B). The increased intracellular insulin level and insulin release of Pax4+ cells in comparison to wt cells correlated with higher levels of C-peptide expression in Pax4+ cells.

Insulin-secreting cells generated from Pax4+ cells normalize blood glucose levels in diabetic mice

We tested the *in vivo* potential of ES-derived insulin-producing cells by transplanting differentiated (stage 5+28d) wt and Pax4+ cells (n = 4 - 6 x 106), respectively, under the kidney capsule and into the spleen of streptozotocin (STZ)-induced diabetic mice. Cells were transplanted into hyperglycemic mice with blood glu-

cose levels above 15 mM and blood glucose levels and body weight were monitored over a period of about 5 weeks. Mice transplanted with wt cells and sham-transplanted control animals remained hyperglycemic at levels of about 25- 30 mM (Fig. 6A) and suffered from diabetic weight loss (Fig. 6B). Animals transplanted with Pax4+ cells reduced blood glucose levels 2 weeks after transplantation and attained a normal glycemia status by 5 weeks (Fig. 6A). Weight loss was attenuated in mice engrafted with Pax4+ cells (Fig. 6B).

In two mice engrafted with Pax4+ cells, partial graft removal by unilateral nephrectomy was performed 26 days after transplantation. These mice showed an increase in blood glucose level in contrast to non-nephrectomized mice (Fig. 6A). Some mice transplanted either with wt or Pax4+ cells developed tumours.

Discussion

The application of a new protocol to generate insulin-producing cells without selecting for nestin-positive cells enabled the formation of islet-like clusters that express Pdx1, Pax4, IAPP and insulin. The cells released insulin in response to glucose, showed beta-cell-specific ion channel activity and are able to rescue diabetes in an animal model. C-peptide expression in insulin-producing cells and glucose-dependent insulin release presents evidence that the cells produced and released insulin, whereas only a low fraction of cells may uptake insulin from the medium. We show that the differentiation of ES cells without selection of nestin-positive cells generates islet-like clusters. In principle, the formation of insulin-producing cells was described by several authors (Lumelsky *et al.*, 2001; Hori *et al.*, 2002; Blyszczuk *et al.*, 2003; Moritoh *et al.*, 2003; Miyazaki *et al.*, 2004), however, the specific properties of ES-derived pancreatic cells may be different. It was presented that insulin-positive cells derived from ES cells (*via* selecting nestin-positive progenitors!) concentrate insulin from the medium (Rajagopal *et al.*, 2003; Sipione *et al.*, 2004) and/or that the differentiated cells show an artifactual insulin release (Hansson *et al.*, 2004). These findings would suggest that pancreatic differentiation from ES-derived nestin-positive cells generated by selective culture in ITSFn medium and in the presence of bFGF is irreproducible and shows no or very limited evidence of pancreatic differentiation.

Our data show that the selection of nestin-positive cells with ITSFn and bFGF is neither obligatory nor profitable for successful pancreatic differentiation and does not promote the generation of specific pancreatic progenitors when applied to ES-derived cells. Previously, we have shown that the expansion of nestin-positive cells in the presence of bFGF is not required for the activation of pancreatic differentiation [(Blyszczuk *et al.*, 2003), see Fig. 1B]. Therefore, in our present study, all selective steps, which support the growth and maintenance of nestin-positive cells, were avoided. Nevertheless, we show that nestin-expressing cells develop *in vitro* from ES cells following the induction of pancreatic differentiation.

Nestin is unequivocally accepted as a marker of neural progenitor cells, however the expression of nestin during pancreatic development remained controversial. Nestin was found in a subset of (insulin-negative) cells in pancreatic islets, suggesting that it might be a marker of pancreatic progenitor cells (Hunziker and Stein, 2000); however, Selander and Edlund did not find nestin in the pancreatic ductal epithelium (Selander and Edlund, 2002), where the potential progenitor cells reside (Bouwens, 1998; Bonner-Weir *et al.*, 2000), instead, nestin was detected in mesenchymal cells, called pancreatic stellate cells (Lardon *et al.*, 2002; Klein *et al.*, 2003). Recent lineage-tracing studies using *nestin* regulatory elements to drive Cre-mediated activation of reporter genes in the developing pancreatic epithelium showed that nestin was transiently expressed in pancreatic epithelial progenitor cells in E10.5 mouse embryos (Esni *et al.*, 2004). Later in development, nestin expressing cells were found to contribute mainly to the exocrine lineage (Delacour *et al.*, 2004). In human pancreatic tissue, nestin was detected only at low levels in endocrine cells (Street *et al.*, 2004). But, when cultivated *in vitro*, islet-derived cells express nestin, proliferate and differentiate into pancreatic endocrine phenotypes (Zulewski *et al.*, 2001). Recent data confirmed that isolated and *in vitro* cultured adult pancreatic progenitors co-express neural and pancreatic markers including nestin (Seaberg *et al.*, 2004). Here, we show that nestin is transiently expressed in ES-derived pancreatic cells. After induction of pancreatic differentiation, nestin is highly co-expressed with C-peptide at an intermediate stage, but at the terminal stage, C-peptide positive clusters were negative for nestin. These results show that *in vitro*, nestin is transiently expressed in ES-derived pancreatic progenitors committed to differentiation, similar to ES-derived neuronal [see Lee *et al.* (2000); Rolletschek *et al.* (2001)] and hepatic [see Kania *et al.* (2003)] lineages, but nestin is not detected in terminally differentiated pancreatic (as well as differentiated neuronal and hepatic) cell types. Moreover, before induction of pancreatic differentiation, a fraction of cells co-expressing nestin and desmin was found. Together, these data suggest that nestin expression in ES-derived progeny represents a property of

TABLE 3

ELECTROPHYSIOLOGICAL PARAMETERS OF WT AND PAX4+ ES-DERIVED CELLS (N= 16) FROM ISLET-LIKE CLUSTERS AT STAGE 5+30D

Parameter	Wt cells		Pax4⁺ cells		t-test	embryonic β-cells		adult β-cells
	amplitude	n	amplitude	n	P≥	amplitude	n	amplitude
Cell capacitance (pF)	4.7 ± 0.6	14	6.7 ± 0.9	11	0.08	3.63 ±0.33	10	7.4 ± 0.3
Vm (start) (mV)	-37.8 ± 3.2	12	-39.9 ± 2.8	14	0.61	-50.5 ±7.6	6	-60/ -80
Na^+ current (pA)	-1310 ± 511	6/15	-938 ± 317	7/11	0.42	-365 ±31	4/9	-392 ± 10
Half inact. Na^+ (mV)	-86.0 ± 5.1	5/6	-84.2 ± 4.1	5/7	0.28	-100 ±7	4	-104 ± 1
K^+steady (pA)	+508 ± 70	13/16	+598 ± 106	14/16	0.34	1176±156	8	+950
Gap-junction conductance (nS)	1.6 ± 0.2	5/5	1.6 ± 0.4	4/5	0.89	0.9 ±0.3	9	1.4 ± 0.2
K_{ATP} conductance (nS)		0/5	0.28 ± 0.06	3/5		1.78 ±0.50	9	2.6
Diazoxide ind.								
ΔVm (mV)	-13	1/5	-12.5 ± 2.1	4/5		38.7 ±5.6	5/5	
Capacitance (fF)	48	1/15	170 ± 95	3/13		30±5	4/6	165±39

Each value represents mean ± SEM. Statistical significance was tested by the Student T-test. The values reported for embryonic beta-cells are from Meneghel-Rozzo *et al.* (2004). The values for adult beta-cells are from Göpel *et al.* (1999) and Speier and Rupnik (2003).

multi-lineage progenitors characterized by immaturity and differentiation capacity (Wiese *et al.*, 2004).

We demonstrate that ES-derived cells at the intermediate stage express C-peptide, CK19, carbonic anhydrase II and partially Isl-1. These properties suggest that the ES-derived progenitor population shows similarities with developing pancreatic beta-cells and pancreatic duct epithelial cells. CK19 is expressed in the duct epithelium in proposed pancreatic stem cells (Bouwens, 1998; Brembeck *et al.*, 2001), but not in functional islets (Bouwens *et al.*, 1997). *In vitro*, CK19 was not organized in intermediate filaments as demonstrated for pancreatic duct cells *in vivo* [see (Bouwens *et al.*, 1995; Bouwens, 1998)]. Functional cytokeratins form heteropolymeric filaments composed of CKs of subclasses I and II (Herrmann and Aebi, 2000). Therefore, we may argue that the CK19 filament organization is defective in ES-derived cells, because of inappropriate intermediate filament association (or lack of intermediate filament associated proteins) due to insufficient or not optimal culture conditions. But, similar to the *in vivo* situation, CK19 is down regulated in ES-derived (C-peptide-positive) islet-like clusters at the terminal differentiation stage. Here, we firstly show that ES-derived pancreatic cells reveal at least, some functional properties of embryonic beta-like cells. The characteristic voltage-activated Na+ channel inactivation (Göpel *et al.*, 1999), voltage-activated K+ channel properties and the Ca+2-dependent secretory activity in ES-derived pancreatic cells suggest a similar excitability of ES-derived pancreatic cells compared to embryonic beta-cells. These cells additionally are characterized by the presence of KATP channels (Ashcroft and Rorsman, 1989) that were found in Pax4+, but not in wt cells. These and the other properties of ES-derived pancreatic islet-like clusters, such as partial expression of CK19 and relatively low insulin levels, demonstrate that the cells are comparable to embryonic beta cells and do not represent definitive islet cells. This would be in agreement with recent data of quantitative gene expression levels in ES-derived hepatocyte-like cells that corresponded to a fetal developmental stage (Jochheim *et al.*, 2004). The rescue of the diabetic phenotype by differentiated Pax4+ cells indicates that ES cells expressing *Pax4* have the potential to function as beta-like cells. The higher efficiency of Pax4+ cells *in vivo* corresponds to the *in vitro* electrophysiological properties showing that Pax4+ cells represent a higher maturation stage in comparison to wt cells. Because also three other independently transfected Pax4+ clones showed increased insulin synthesis (unpublished data), we exclude clonal variation as explanation for the improved functional capacity of Pax4+ cells. Since there was a delay in blood glucose improvement for two weeks after transplantation, we speculate that *in vivo*, engrafted cells would mature further into a more functional cell type with physiological properties comparable to beta-like cells. Such *in vivo* maturation has been also observed in porcine neonatal pancreatic cell clusters transplanted into diabetic mice (Omer *et al.*, 2003). In the future, a more detailed characterization of the transplanted cells will be necessary to assess the influence of the *in vivo* environment on the maturation of insulin-producing cells.

In summary, our model represents a new strategy to generate pancreatic progenitor and early pancreatic endocrine cells by a procedure that avoids all selection steps, which might critically affect ES cell differentiation into insulin-producing cells. Our *in vitro* differentiation protocol allows the investigation of genetic and epigenetic modifications in ES cells as well as the analysis of differentiation factors and signalling mechanisms necessary for the generation and maturation of pancreatic progenitors *in vitro*.

Materials and Methods

Cell culture and differentiation conditions

ES cells of line R1 (wild type, wt) and cells constitutively expressing Pax4 (Pax4+) were cultured as described (Wobus *et al.*, 2002; Blyszczuk *et al.*, 2003). Cells were differentiated in embryoid bodies (EBs, 600 ES cells/EB) using the "hanging drop" method (Wobus *et al.*, 2002). At day 5, EBs were plated in Iscove's modified Dulbecco's medium (IMDM, Gibco, Germany) supplemented with 20% FCS, L-glutamine, non-essential amino acids and α-monothioglycerol [see (Wobus *et al.*, 2002)] and cultured for 9 days. At day 5+9, EB outgrowths were dissociated by 0.1% trypsin (Gibco): 0.08% EDTA (Sigma, Germany) in PBS (1:1) for 1 min, collected by centrifugation and re-plated onto poly-L-ornithine/laminin-coated tissue culture dishes in "N2 medium + NA" containing DMEM/F12 (Gibco) supplemented by 20 nM progesterone, 100 μM putrescine, 1 μg/ml laminin, 25 μg/ml insulin, 30 nM sodium selenite, 10 mM nicotinamide (NA) (all from Sigma), 50 μg/ml transferrin, 5 μg/ml fibronectin, B27 media supplement (dilution 1:50) (all from Gibco) and 15% FCS. At day 5+10, the medium was changed and cells were cultivated in the differentiation medium without FCS until stages of analysis.

Immunofluorescence analysis

ES-derived cells growing on cover slips were fixed by 4% paraformaldehyde in PBS for 20 minutes at room temperature and with methanol: acetone (7:3) for 10 minutes at -20°C, respectively and processed for immunofluorescence analysis as described (Wobus *et al.*, 2002). Hoechst 33342 (5 μg/ml, 10 minutes, 37°C) was used to label the nuclei. The following primary antibodies and dilutions were used: guinea pig IgG anti-C-peptide (Linco, USA) 1:100, mouse IgG anti-nestin (Developmental Studies Hybridoma Bank, USA) 1:3, rabbit IgG anti-desmin (Dako, Denmark) 1:100, mouse IgM anti-cytokeratin19 (Cymbus, UK) 1:100, rabbit IgG anti-Isl-1 (Abcam, UK) 1:200, rabbit IgG anti-carbonic anhydrase II (Abcam) 1:200 and mouse IgG anti-insulin (Sigma) 1:40. The following secondary antibodies were used according to the manufacturer's recommendations: FITC anti-mouse IgG, Cy3 anti-mouse IgG, Cy3 anti-mouse IgM, FITC anti-mouse IgG, FITC anti-guinea pig IgG, FITC anti-mouse IgG, FITC anti-rabbit IgG, Cy3 anti-rabbit IgG (all from Jackson ImmunoResearch Laboratories, USA). Samples were analysed by the fluorescence microscope ECLIPSE TE300 (Nikon, Japan) and the confocal laser scanning microscope LSM 510 META (Carl Zeiss, Germany). For quantitative evaluation of each experiment, at least 1000 Hoechst-positive cells were analysed.

RT-PCR analysis

Cells were suspended in lysis buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7; 0.5% sarcosyl, 0.1 M β-mercaptoethanol). Total RNA was isolated by the single step extraction method according to (Chomczynski and Sacchi, 1987). mRNA was reverse transcribed using PolyT tail primer Oligo d(T)16 (PerkinElmer, Überlingen, Germany) and cDNA was amplified using the following primers: (name of the analysed gene, forward and reverse primer sequence and the length of the amplified fragment are given): *CK19* 5'-ctgcagatgacttcagaacc-3', 5'-ggccatgatctcatactgac-3', 299 bp; *IAPP*, 5'-tgatattgctgcctcggacc-3', 5'-ggaggactggaccaaggttg-3', 233 bp; *insulin*, 5'-gtggatgcgcttcctgcccctg-3', 5'-atgctggtgcagcactga-3', 288 bp; *nestin* 5'-ctaccaggagcgcgtggc-3', 5'-tccacagccagctggaactt-3', 220 bp; *Pax4*, 5'-accagagcttgcactggact-3', 5'-cccatttcagcttctcttgc-3' 300 bp; *Pdx1*, 5'-ctttcccgtggatgaaatcc-3', 5'-gtcaagttcaacatcactgcc-3', 230 bp; *β-tubulin*, 5'-tcactgtgcctgaacttacc-3', 5'-ggaacatagccgtaaactgc-3' 317 bp.

One third of each PCR reaction was electrophoretically separated on 2% agarose gels containing 0.35 μg/ml of ethidium bromide. Gels were illuminated with UV light and the ethidium bromide fluorescence signals of

gels were stored by the E.A.S.Y. system (Herolab GmbH, Wiesloch, Germany).

Electrophysiological measurements

The electrophysiological methods used were similar to those described for primary pancreatic tissue slices (Speier and Rupnik, 2003). Clusters at stage 5+30d were resuspended into low gelling point agarose (Seaplaque GTG agarose, BMA, Walkersville, MD, USA; 1.9% w/v in extracellular solution), cooled and sliced in ice-cold solution using a vibratome (VT 1000 S, Leica, Nussloch, Germany). The slices, 100 μm thick, were stored at room temperature in extracellular solution bubbled with carbogen (95% O2, 5% CO2). Standard whole-cell patch-clamp was used in current- and in voltage-clamp mode to measure the following electrophysiological parameters: membrane potential and membrane capacitance, currents through voltage-activated Na+ and K+ channels and ATP-sensitive K+ (KATP) channels. Glucose (20 mM) and classical agonists and antagonists of insulin secretion [tolbutamide (100 uM) and diazoxide (200 uM)] were used to modify the electrical properties of the cells. A patch-clamp lock-in amplifier (SWAM II, Celica, Ljubljana, Slovenia) operating at 1.6 kHz lock-in frequencies was used. Data were transferred to a PC via an A/D converter (PCI-6035E, National Instruments, Austin, Tex., USA) and acquired by WinWCP software (John Dempster, University of Strathclyde, UK). Borosilicate glass capillaries (GC150F-15; WPI, Sarasota, Fla., USA) were pulled (P-97; Sutter Instruments, Novato, CA, USA) to a resistance of 3–4 MΩ in our pipette solution. During patch-clamp experiments, the slices were superfused at 35°C with extracellular solution and held on the bottom of the recording chamber by a platinum-frame with nylon fibre. The perfusion chamber was mounted on an inverted microscope (40w, NA 0.8 and 60w, NA 1.0, Eclipse E600FN; Nikon, Japan). The extracellular solution contained (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH2PO4, 26 NaHCO3, 2 Na-pyruvate, 0.5 ascorbic acid, 3 myo-inositol, 5 lactic acid, 1 MgCl2, 2 CaCl2, 3 glucose. The pipette solution contained (in mM): 150 KCl, 10 HEPES, 2 MgCl2 x 6H2O, 0.05 EGTA, 2 ATPNa2, pH 7.2 (adjusted with KOH) (all from Sigma).

Quantitative insulin determination by ELISA

To estimate total cellular and secreted insulin levels, differentiated wt and Pax4+ cells were pre-incubated for 90 minutes at 37ºC in Krebs Ringer Bicarbonate Hepes (KRBH) buffer containing 118 mM sodium chloride, 4.7 mM potassium chloride, 1.1 mM potassium dihydrogen phosphate, 25 mM sodium hydrogen carbonate (all from Carl Roth), 3.4 mM calcium chloride (Sigma), 2.5 mM magnesium sulphate (Merck, Germany), 10 mM Hepes (Gibco) and 2 mg/ml bovine serum albumin (Gibco) supplemented with 2.5 mM glucose. For induced insulin release, cells were further incubated in KRBH buffer supplemented with 27.7 mM glucose and alternatively with 5.5 mM glucose and 10 μM tolbutamide (Sigma) for 15 minutes at 37°C. The control was incubated with 5.5 mM glucose. Proteins were extracted from the cells with acid ethanol at 4°C overnight, followed by cell sonification. Determination of cellular and secreted insulin was performed using an insulin ELISA kit (Mercodia AB, Sweden). Protein was determined by the Bradford assay (Bio-Rad).

Transplantation into Streptozotocin (STZ) induced diabetic mice

Transplantations were essentially carried out as described (Blyszczuk *et al.*, 2003). In brief, 8-10 week old male BALB/c mice (n = 25 each for wt and Pax4+ cells, n = 14 for sham transplantation) were treated with a single i.p. injection of 200 mg/kg body weight STZ (Sigma). The diabetic status was determined by the presence of weight loss, polyuria and hyperglycaemia. For surgery, mice were anaesthetized with avertin (2.5 % in PBS, 15 μl/ g body weight). Through a lumbar incision, the left kidney and the spleen were exposed. Using a blunt 30g-needle, approximately 4-6 x 106 cells were injected under the kidney capsule and into the spleen. Alternatively, culture medium was applied for sham transplants as control. Non-fasted blood glucose levels were measured between 8:00 and 10:00 a.m. using a OneTouch glucometer. For unilateral nephrectomy, the ureter and all vessels connecting to the left kidney were ligated using 5-0 size suture and the whole kidney was removed from the anaesthetized animal. After 39 days, n= 10 Pax4+-cell-transplanted animals were alive. Out of these, 5 mice responded to transplantation with a normalization of blood glucose levels, as shown in Fig. 6. In contrast, from n= 11 surviving mice that were transplanted with wt cells and n= 8 surviving sham-transplanted mice, none showed improvement of blood glucose levels. Mice of each of these control groups (n= 6) were chosen on the basis of comparable hyperglycaemic status prior to transplantation.

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Chapter 9

In vitro differentiation of mouse ES cells into pancreatic and hepatic cells

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1. INTRODUCTION

Pancreas and liver cells are derivatives of the definitive endoderm. The pancreas develops from dorsal and ventral regions of the foregut, whereas the liver originates from the foregut adjacent to the ventral pancreas compartment (1,2). Pluripotent embryonic stem (ES) cells of the mouse have the capacity for self-renewal and the potential to differentiate into virtually any cell type of the somatic and germ cell lineages, including endodermal cells of pancreas and liver [for reviews see (3,4)]. During *in vitro* differentiation, ES cells show hepatic-restricted (5-7) and pancreatic-restricted (8-10) transcripts and proteins. Therefore, mouse ES cells provide an *in vitro* model system in which to elucidate these differentiation processes.

Differentiation of ES cells without the application of lineage-specific differentiation-inducing factors results in ~~a~~ heterogeneous derivatives and a low yield of endocrine pancreatic (11) and hepatic (12) cells. However, using specific growth and extra-cellular matrix (ECM) factors (7,9) and/or transgene expression (8,10,13)**,** the number of differentiated cell types is enhanced significantly and results in the generation of functional pancreatic and hepatic cells.

At present, the lack of methods to enrich pancreatic or hepatic progenitor cells *in vitro* is a major obstacle for the generation of selected and pure populations of pancreas or liver cells at high efficiency. As yet, the progenitor cells of liver and pancreas are not clearly defined. For example, nestin-positive cells derived from pancreatic islets generated after *in vitro* culture are suggested to represent pancreatic progenitors (14), and oval cells are regarded as hepatic stem/ progenitor cells (15). In addition, cells residing in the pancreatic (16) and hepatic (17) ductal epithelium expressing cytokeratin 19 have been proposed as pancreatic and hepatic stem or progenitor cells *in vivo*.

Here, we describe efficient procedures for the induction of differentiation of mouse ES cells into pancreatic and hepatic lineages. The overall strategy involves (i) the generation of a pool of progenitor cells, including endodermal precursors, by spontaneous differentiation of

ES cells in EBs, and (ii) further, directed differentiation of those progenitor cells into pancreatic and hepatic cell types using lineage-specific, differentiation-inducing and ECM-associated factors. In addition, constitutive over-expression of the pancreatic developmental control gene, *Pax4*, may be induced in ES cells to increase the number of functional islet-like cells (10).

2. CULTURE OF UNDIFFERENTIATED ES CELLS

Reproducibility in ES cell differentiation experiments is strongly dependent on a high standard of culture of the ES cells used. In order to maintain ES cells in the undifferentiated state, they must be cultured at relatively high density and in the presence of mouse embryonic fibroblast (MEF) feeder cells and/or leukemia inhibitory factor (LIF) (see chapters 1 and 2). Good quality foetal calf serum (FCS) is critical for long-term culture of ES cells and for their subsequent differentiation. As mouse ES cells divide every 12– 15 h, the culture medium should be replenished daily and the cells passaged every 24 – 48 h on freshly-prepared, mitotically-inactivated feeder layers [see chapter 2, and (18)]. For passaging, ES cells must be dissociated carefully by treatment with trypsin-EDTA solution. If one or more of these requirements are not complied with, ES cells may differentiate spontaneously during culture and become unsuitable for differentiation studies.

Protocol 1

Routine subculture of mouse ES cells

Reagents

- ES cell medium: Dulbecco's modification of Eagle's liquid medium (DMEM, 4.5 g/l glucose, Invitrogen, cat. no. 52100-047) supplemented by sterile additives; 2 mM L-glutamine (Invitrogen, cat. no. 25030-024), non-essential amino acids (Invitrogen, cat.

no. 11140-035), 100 µM beta-mercaptoethanol (Serva, cat. no. 28625), 0.05 mg/ml streptomycin, 0.03 mg/ml penicillin (both Invitrogen, cat. no. 15070-063), 15% foetal calf serum (Invitrogen, cat. no. 10207-106), 1,000 units/ml leukemia inhibitory factor (LIF, Chemicon cat. no. ESG1106). An alternative formulation for ES cell medium is provided in Chapter 2, Protocol 1.

- MEF feeder layers in gelatinised 60 mm culture dishes (see Chapter 2, Protocols 2, 3).
- Phosphate-buffered saline (PBS): 10 g NaCl, 0.25 g KCl, 1.44 g Na_2HPO_4, 0.25 g $KH_2PO_4.2H_2O$. Dissolve in 1 l distilled water and sterilize through 0.22 µm filter.
- Trypsin-EDTA solution: prepare 0.2 % (w/v) trypsin (Serva, cat. no. 37290) in PBS and 0.02 % (w/v) EDTA (Sigma, cat. no. E-6758) in PBS. Sterilize through 0.22 µm filter. Mix trypsin and EDTA solutions 1:1. Prepare fresh every week.

Method

1. Culture ES cells on MEF feeder layers in gelatinised tissue culture dishes in ES cell medium.
2. Change the medium 1 to 2 h before passage.
3. Aspirate the medium and quickly rinse the dish with 2 ml of trypsin-EDTA solution to remove residual serum-containing medium.
4. Add 2 ml of trypsin-EDTA and quickly aspirate the solution leaving an amount sufficient to cover the cell monolayer (up to 200 µl) and incubate at r.t. for 30 to 60 s.
5. Add 2 ml fresh ES cell medium to the cell monolayer
6. Resuspend the cell population using a 2 ml glass pipette into a single cell suspension, and split 1: 3 into to freshly prepared feeder plates.

3. DIFFERENTIATION PROCEDURES

3.1. Generation of ES-derived progenitor cells

In the absence of feeder cells and LIF, ES cells start spontaneously to differentiate.

The controlled production of lineage progenitors cells entails two consecutive steps: (i) ES cells are cultured to form three-dimensional aggregates called “embryoid bodies” (EBs), to promote differentiation into early progenitors of all lineages; (ii) EBs are plated onto adhesive substrates to allow expansion and further differentiation of the progenitor cells. ES cells may be cultured in EBs either by the “hanging drop” method (18) or by "mass culture" in bacteriological plates (19). The advantage of the “hanging drop” method used here is a low variation in the size of EBs, due to a controlled number of ES cells in the starting aggregates and also higher reproducibility of differentiation. EB formation for 5 d allows the development of cells of all three primary germ layers.

Protocol 2

Generation of EBs by the “hanging-drop” method

Reagents

- Differentiation Medium I: Iscove’s modification of DMEM (IMDM, Invitrogen, cat. no. 42200-030) supplemented with sterile additives; 20% foetal calf serum (Invitrogen, cat. no. 10207-106), 2 mM L-glutamine (Invitrogen, cat. no. 25030-024), non-essential amino acids (Invitrogen, cat. no. 11140-035), 450 µM monothioglycerol (3-mercapto-1,2-propanediol, Sigma, cat. no. M-6145), 0.05 mg/ml streptomycin, 0.03 mg/ml penicillin (both Invitrogen, cat. no. 15070-063).
- 0.1% gelatin: 0.1 % (w/v) gelatin (Fluka, cat. no. 48720) in distilled water, sterilized by autoclaving.

Method

1. Trypsinise ES cells as in Protocol 1 and re-suspend the single cells in 5ml/60 mm dish of Differentiation Medium I.
2. Adjust the cell density in the suspension to contain 30,000 cells/ml, i.e. 600 cells in 20 µl of Differentiation Medium I.
3. Place single drops (n= 50 to 60) of 20 µl of the ES cell suspension onto the underside of lids of 100 mm bacteriological petri dishes, each containing 10 ml PBS, and replace the lids onto the plates. The drops of cells are now hanging upside-down from the lids and over the PBS.
4. Incubate the ES cells in these hanging drops for 2 d, during which time the cells aggregate and form one EB per drop. Monitor the development of EBs by light microscopy.
5. Take up the EBs carefully from the lids in 2 ml Differentiation Medium I, and transfer into 60 mm bacteriological petri dishes containing an additional 5 ml of Differentiation Medium I, and continue incubation in suspension for 3 d. (EBs fail to adhere to the bacteriological-grade plastic).

After 5 d in suspension culture, EBs must be plated onto an adhesive surface, at a proper density and with homogeneous distribution. The distance between individual EBs should be sufficient to allow the differentiating cells to proliferate and to migrate for several days after plating. If the EBs are plated at too high a density, differentiation may be inhibited. Continued culture of EB outgrowths results in multi-layered cell clusters.

Protocol 3

Differentiation of EBs on adhesive substratum

Method

1. Prepare gelatin-coated (60 mm) tissue-culture plates by adding a sufficient amount of 0.1% gelatin to cover the bottom surface of the dish, and incubate either for 1 h or overnight at 4°C. For immunofluorescence analysis, place cover slips on the bottom of the dish before gelatin coating. Aspirate the gelatin solution before use.
2. Add a sufficient amount of Differentiation Medium I (=5 ml/60 mm dish), and transfer EBs onto the tissue-culture plates. Plate 20 to 30 EBs per 60 mm dish, and 5 to 10 EBs per 35 mm dish, respectively. For morphological analysis of EB outgrowths, transfer one EB per well of a 24-well micro-well plate.
3. Change the medium every second to third day until 9 days after EB plating (= 5+9 d in total from aggregation).

3.2. Induction of lineage-specific differentiation

Spontaneous differentiation of ES cells results in the generation of cellular derivatives of all three primary germ layers, but the proliferation and differentiation of specific progenitor cells is promoted by culture in the presence of specific growth and differentiation-inducing factors. The following parameters affect the efficiency of differentiation of ES-derived progenitors into pancreatic and hepatic cells:

(i) Dissociation of the compact structure of EB outgrowths. Cell-to-cell interactions within EB outgrowths may influence the fate of progenitor cells.

(ii) A suitable adhesive surface. ECM factors determine adhesion, proliferation and migration of specific progenitor cells after re-plating of EB-dissociated cells.

(iii) Cell density after re-plating. To support the proliferation and migration of a specific progenitor population, the concentration of cells should be optimal to prevent overgrowth resulting in metabolic starvation, necrosis and cell death. However, if the initial density of cells is too low, the level of autocrine factors may not reach a threshold level. Consequently,

the low growth factor activity and reduced cell-to-cell contacts may result in poor differentiation of specific cell types.

3.2.1. Induction of pancreatic differentiation

The differentiation medium used for the generation of pancreatic cells contains factors required for cell survival, such as progesterone, putrescine, insulin, sodium selenite and transferrin, as well as factors promoting pancreatic differentiation, such as nicotinamide (20) and laminin (21). The pancreatic differentiation medium is serum-free. Coating of the tissue-culture plates with poly-L-ornithine/laminin is necessary for pancreatic differentiation. The addition to the medium of other substances promoting pancreatic differentiation, such as glucagons-like peptide-1 [GLP-1, (22)] and growth inhibitors (23), may be helpful. After re-plating and culture of EBs for 7 d, pancreatic progenitor cells with a characteristic morphology (Figure 1 A) appear. Continued differentiation results in the formation of typical cell clusters (Figure 1 C).

Protocol 4

Preparation of poly-L-ornithine/laminin-coated dishes

Reagents

- Sodium borate buffer: 10 mM H_3BO_3 in distilled water. Adjust to pH 8.4 with NaOH.
- poly-L-ornithine solution: 0.1 mg/ml poly-L-ornithine (Sigma, cat. no. P-2533) in sodium borate buffer. Sterilize through 0.22 μm filter.
- laminin solution: 0.001 mg/ml laminin (Sigma, cat. no. L-2020) in sterile PBS.

Method

1. Add sterile poly-L-ornithine solution to the culture dishes (sufficient to cover the

bases) and incubate for 3 h at 37°C. For immunofluorescence analysis, place cover slips on the bottom of the dishes before adding poly-L-ornithine solution.

2. Aspirate the poly-L-ornithine solution, wash 3 times with distilled water, and incubate with 5 ml distilled water at room temperature for 12 h.
3. Rinse the dishes 3 times with distilled water and dry at 40°C.
4. Now incubate the poly-L-ornithine-coated dishes with laminin solution at 37°C for 3 h.
5. Aspirate the laminin solution and rinse the dishes 2 times with PBS.

Protocol 5

Induction of pancreatic differentiation

Reagents

- 5+9 d adherent cultures of EBs in tissue-culture dishes (Protocol 3)
- PBS
- Trypsin-EDTA solution
- Cell scraper (Greiner bio-one, cat. no. 541070)
- Pancreatic differentiation medium (PDM): liquid medium DMEM/F12 (4.5 g/l glucose, Invitrogen, cat. no. 32500-043) supplemented with sterile additives (from Sigma, unless otherwise indicated); 20 nM progesterone (cat. no. P-7556), 100 µM putrescine (cat. no. P-5780), 1 µg/ml laminin (cat. no. L-2020), 10 mM nicotinamide (cat. no. N-3376), 25 µg/ml insulin (cat. no. I-1882), 30 nM sodium selenite (cat. no. S-5261), 50 µg/ml transferrin (cat. no. T-1147), B27 media supplement (Invitrogen, cat. no. 17504-044), 0.05 mg/ml streptomycin and 0.03 mg/ml penicillin (both Invitrogen, cat. no. 15070-063)
- Foetal calf serum (Invitrogen, cat. no. 10207-106)

- Sodium borate buffer
- Poly-L-ornithine/laminin-coated 60 mm tissue-culture dishes.

Method

1. Aspirate the medium from 60 mm tissue-culture plates containing differentiating EB outgrowths, and quickly rinse the cultures with 2 ml of PBS.
2. Add 2 ml of trypsin-EDTA and incubate at r.t. for 1 min.
3. Aspirate trypsin-EDTA completely and gently mechanically detach cells with the cell scraper.
4. Add 4 ml of freshly prepared Pancreatic Differentiation Medium supplemented with 10% FCS.
5. Resuspend the cells with a 5 ml glass pipette to obtain a suspension of single cells and small clusters, and plate 1 ml onto four freshly-prepared poly-L-ornithine/laminin-coated 60 mm tissue-culture dishes.
6. Add 3 ml PDM supplemented with 10% FCS, and incubate overnight.
7. The next day check that all cells are attached, aspirate the medium, and add PDM without FCS.
8. Change the medium every second to third day, and also one day prior to analysis.

To increase the number of ES-derived pancreatic cells, we recommend the use of transgenic ES cells that constitutively express pancreatic developmental control genes. We have shown that over-expression of *Pax4* in the R1 line of mouse ES cells significantly up-regulates pancreatic beta cell-specific mRNA and protein levels, resulting in an increased number of insulin-expressing cells (10).

3.2.2. Induction of hepatic differentiation

Mouse ES cells are induced to differentiate into hepatic cells when incubated in Hepatocyte

Culture Medium (HCM). HCM contains factors required for cell survival, such as insulin and transferrin, as well as hepatic differentiation factors, including hydrocortisone and epidermal growth factor (EGF). Addition of 10% FCS to HCM is required for cell survival. The culture plates are coated with collagen type I (collagen I), which further promotes hepatic differentiation (5). After re-plating EBs in HCM plus 10% FCS, hepatic progenitor cells develop with characteristic morphology (Figure 1 B). With further incubation and differentiation, large cuboidal, epithelial-like (partially bi-nucleated) hepatocyte-like cells (Figure 1 D) appear.

Protocol 6

Preparation of collagen I-coated dishes

Reagents

- 0.02 N acetic acid solution in distilled water. Sterilize through 0.22 µm filter.
- Collagen I solution: 0.05 mg/ml Collagen type I (BD Biosciences, cat. no. 354236) in 0.02N acetic acid.
- Tissue-culture plates (60 mm)

Method

1. Add 5 ml sterile collagen I solution to 60 mm culture plates and incubate at r.t. for 1 h. For immunofluorescence analysis, place cover slips on the bottom of the culture plate before adding of the collagen I solution.
2. Aspirate collagen I solution, and wash dishes 2 times with PBS.

Protocol 7

Induction of hepatic differentiation

Reagents

- 5+9 d adherent cultures of EBs in 60 mm tissue-culture dishes (Protocol 3)
- PBS
- Trypsin/EDTA solution
- Cell scraper (Greiner bio-one, cat. no. 541070)
- Hepatic culture medium (HCM): Hepatocyte basal, modified Williams 'E medium (HBM medium, Cambrex, cat. no. CC-3199) containing the following quantities of sterile additives per 500 ml (all from Cambrex, unless otherwise indicated); 0.5 ml ascorbic acid (cat. no. CC-4316), 10 ml bovine serum albumin fatty-acid-free (cat. no. CC-4362), 0.5 ml hydrocortisone (cat. no. CC-4335), 0.5 ml transferrin (cat. no. CC-4313), 0.5 ml insulin (cat. no. CC-4321), 0.5 ml human epidermal growth factor (cat. no. CC-4317), 0.5 ml gentamycin-amphothericin (cat. no. CC-4381), 10% foetal calf serum (Invitrogen), 0.05 mg/ml streptomycin and 0.03 mg/ml penicillin (both Invitrogen, cat. no. 15070-063).
- Collagen I-coated 60 mm tissue-culture dishes (Protocol 6)

Method

1. Aspirate the medium from 60 mm culture plates containing the EB outgrowths (see 3.1), quickly rinse the cells with 2 ml PBS to remove residual medium.
2. Add 2 ml of trypsin-EDTA and incubate at r.t. for 1 min.
3. Aspirate trypsin-EDTA completely and gently mechanically detach cells with cell scraper.
4. Add 4 ml of freshly prepared Hepatic Culture Medium supplemented with 10% FCS.

5. Resuspend the cells with a 5 ml glass pipette to obtain a suspension of single cells and small clusters and transfer 1 ml into each of four freshly prepared collagen I-coated 60 mm plates.
6. Add 3 ml HCM supplemented with 10% FCS.
7. Change the medium every second to third day, and additionally one day prior to analysis.

4. CHARACTERIZATION OF DIFFERENTIATED PANCREATIC AND HEPATIC PHENOTYPES

We present three basic methods for characterising the differentiation status of ES-derived pancreatic and hepatic cells: (i) by reverse transcriptase-polymerase chain reaction (RT-PCR), (ii) by immunofluorescence staining and (iii) by enzyme-linked immunosorbent assay (ELISA). These methods allow the analysis and quantification of pancreatic and hepatic differentiation at the transcript and protein levels, as well as the determination of pancreatic- and hepatic-specific markers.

4.1. RT-PCR

A criterion for successful pancreatic and hepatic differentiation of ES cells *in vitro* is the expression of genes that are normally involved in pancreas and liver development *in vivo.* Detailed protocols for RNA isolation, reverse transcription, cDNA amplification and separation of PCR products from ES-cell-differentiated derivatives are described (18). The oligonucleotide sequences specific for selected genes involved in the development and function of pancreatic beta cells and hepatocytes are presented in Table 1. Transcripts of some of the genes may be detected in ES cells and/or ES-cell derivatives after spontaneous

differentiation [(10); Kania *et al.,* unpublished data]. We recommend expression analysis of the following genes as specific markers to denote pancreatic differentiation: insulin, islet amyloid pancreatic polypeptide (IAPP), Pax4 and Pdx1; and for hepatic differentation: albumin, Cyp2b9, Cyp2b13 and tyrosine aminotransferase (TAT).

4.2. Immunofluorescence staining

Immunoflorescence analysis allows the investigation and characterisation of ES cell differentiation in terms of proteins expressed, at both intracellular (e.g. intermediate-filament proteins) and extracellular (e.g. cell-surface antigens) levels.

The method is especially valuable in ES cell differentiation studies, because ES cells often result in a heterogeneous population with respect to cell lineage and developmental stage; and immunofluorescence allows the detection of even a small minority of antigen-positive cells against a largely negative background.

Here, we describe two sample fixation methods using (i) methanol:acetone (suitable for cytoskeletal proteins, such as intermediate filaments) and (ii) paraformaldehyde (suitable for intracellular proteins). The antibodies, dilutions and appropriate fixation methods suitable for pancreatic and hepatic markers are given in Table 2.

Protocol 8

Fixation with methanol: acetone:

Equipment and Reagents

- Blunt forceps
- PBS
- Tissue paper

- Methanol/acetone fixation solution: 7 ml methanol and 3 ml acetone. Mix and store at -20°C.
- Humidified chamber
- 200 ml beaker

Method

1. Remove cover slips containing ES-derived cells (handling by the edge, using blunt forceps) from culture plates and carefully put the edge in contact with tissue paper to drain residual medium.
2. Rinse cover slips twice with PBS in a 200 ml beaker by dipping and gently stirring for 30 s and drain residual PBS.
3. Fix cells onto cover slips by overlaying with 200 µl of methanol/acetone solution (pre-cooled at -20°C), and incubate at -20°C for 10 min.
4. Pick up cover slips and drain the fixative, as before.
5. Wash cover slips three times by dipping and gently stirring in a beaker containing PBS for 30 s.
6. Incubate cover slips in a humidified chamber, overlay with 200 µl PBS at r.t. for 5 min to re-hydrate the samples.
7. The cells are now ready for immunostaining (Protocol 10).

Protocol 9

Fixation method with paraformaldehyde

Reagents

- Blunt forceps
- PBS
- Tissue paper

- Paraformaldehyde fixation solution: 0.4 g paraformaldehyde and 10 ml PBS. Heat the mixture to 60^{o}C, stir until the solution becomes clear, and cool to room temperature (prepare shortly before use).
- Humidified chamber
- 200 ml beaker

Method

1. Collect and wash cover slips as in steps 1 and 2, Protocol 8.
2. Fix cells onto cover slips by overlaying with 200 µl 4% paraformaldehyde in PBS, and incubate in a humidified chamber at r.t. for 15 min.
3. Drain fixative from cover slips, as before.
4. Wash cover slips three times in PBS, as before.
5. The cells are ready now for immunostaining (Protocol 10).

Note: The endogenous level of a particular protein may be over-estimated if that protein is present also in the culture medium. As a case in point, ES-derived pancreatic cells were found to be overly immuno-positive for insulin as a consequence of insulin uptake from the medium (24). Therefore, for pancreatic differentiation, C-peptide, a by-product of insulin synthesis, serves as reliable marker of insulin production. For hepatic differentiation, as albumin is a constituent of the hepatic differentiation medium (see Protocol 7), immuno-reactivity for albumin must be confirmed by co-expression with other hepatic markers. Demonstration of pancreatic or hepatic differentiation by immunofluorescence requires parallel RT-PCR analysis, including a comparison of diverse markers.

Protocol 10

Immunostaining

Reagents and equipment

- 10 % serum solution: 1 ml heat-inactivated serum plus 9 ml PBS. Mix and store at 4°C. (Do not use serum from the same species as has been used for the preparation of the primary antibody!).
- PBS
- Hoechst 33342 solution: 50 µg Hoechst 33342 in 10 ml PBS, giving 5 µg/ml. Mix and store in the dark at 4°C.
- Mounting medium: Vectashield (Vector, cat. no. L-010)
- Humidified chamber
- Fluorescence or confocal microscope. The confocal microscope used here was model LSM 510 META (Carl Zeiss, Germany).
- 200 ml beaker

Method

1. To prevent unspecific binding, incubate cover slips with fixed cells with 10 % serum solution (apply ~100 µl overlay per cover slip) in a humidified chamber at r.t. for 30 min.
2. Prepare 60-100 µl/sample of the primary antibody by dilution in PBS (the optimal dilution has to be determined separately).
3. Incubate cover slips with the primary antibody in a humidified chamber at 37°C for 60- 90 minutes.
4. Rinse cover slips three times with PBS in a 200 ml beaker by dipping and gently stirring for 30 s.
5. Prepare 100 µl/sample of the secondary, fluorescence-conjugated antibody by dilution in PBS (the optimal dilution has to be determined separately).
6. Incubate cover slips in a humidified chamber with the secondary antibody at 37°C for 45 min.

7. Rinse cover slips three times with PBS at r.t, as in step 4.
8. For counter-staining of nuclei, incubate cells on cover slips with 200 µl Hoechst 33342 solution in a humidified chamber at 37°C for 5 min.
9. Rinse cover slips three times with PBS at r.t, as in step 4 and additionally quickly with distilled water.
10. Embed cells on cover slips in mounting medium and analyse samples using a fluorescence or confocal microscope.

4.3. ELISA

The Enzyme-Linked Immunosorbent Assay (ELISA) is a common immunoassay used for the detection and quantification of a substance based on immunological reactions. ELISA is a valuable tool in ES cell technology, because the technique is highly sensitive, specific and allows rapid and simultaneous processing of a large number of samples. Here, we describe methods suitable for the quantification of secreted and intracellular insulin and of secreted albumin in ES cell culture systems.

Protocol 11

Insulin ELISA:

Equipment and Reagents

- Krebs Ringer Bicarbonate Hepes (KRBH) buffer: 118 mM sodium chloride, 4.7 mM potassium chloride, 1.1 mM potassium dihydrogen phosphate, 25 mM sodium hydrogen carbonate, 3.4 mM calcium chloride, 2.5 mM magnesium sulphate, 10 mM Hepes and 2 mg/ml bovine serum albumin
- Glucose

- Dulbecco's modification of Eagle's liquid medium (DMEM, 4.5 g/l glucose, Invitrogen, cat. no. 52100-047) supplemented by 10% foetal calf serum (Invitrogen, cat. no. 10207-106)
- Trypsin-EDTA solution
- 15 ml PP-test tubes (Cellstar, cat. no. 188271)
- 1.5 ml tubes (Eppendorf AG, cat. no. 0300 121.848)
- Acid ethanol: mix 1 ml hydrochloric acid and 9 ml absolute ethanol
- Ultrasonic homogenizer (Sonoplus HD70, Bandelin)
- Insulin enzyme-linked immunosorbent assay (Mercodia AB, cat. no. 10-1149-01)
- Protein Bradford Assay (Bio-Rad Laboratories, cat. no. 500-0006).

Method

1. Aspirate the medium from two 35 mm tissue-culture plates containing ES-derived pancreatic cells and rinse the cultures 5 times with 3 ml of PBS.
2. Replace PBS with freshly prepared KRBH buffer supplemented with 2.5 mM glucose and incubate for 90 minutes at 37°C.
3. Aspirate KRBH buffer supplemented with 2.5 mM glucose and add to separate tissue-culture plates KRBH buffer supplemented with either 5.5 mM glucose or 27.7 mM glucose, and incubate for 15 min at 37°C.
4. Collect supernatant and store at –20°C for determination of insulin release.
5. Add a sufficient amount of trypsin-EDTA solution to cover the whole surface and incubate at r.t. for 2-3 min.
6. Remove carefully the trypsin-EDTA solution, add 3 ml of DMEM medium supplemented with 10% FCS.
7. Resuspend the cells with a 2 ml glass pipette and transfer to 15 ml tubes.
8. Centrifuge for 5 min at 1000 g at r.t.

9. Aspirate supernatant, resuspend cells in 50 µl acid ethanol, transfer to 1.5 ml tubes and incubate overnight at 4°C.
10. Disintegrate cells using ultrasonic homogenizer according to manufacturer recommendations.
11. Add 500 µl distilled water, mix and centrifuge for 1 min at 13 000 g at r.t.
12. Transfer supernatant to new 1.5 ml tubes and store at –20°C for determination of intracellular total protein and insulin level.
13. Determine insulin level with ELISA according to manufacturer recommendations.
14. Determine total protein level with protein Bradford assay according to manufacturer recommendations.

Protocol 12

Albumin ELISA:

Equipment and Reagents

- PBS
- HCM medium without BSA and FCS
- Albumin enzyme-linked immunosorbent assay (Bethyl Laboratories, cat. no. E90-134**)**
- 1.5 ml tubes (Eppendorf AG, cat. no. 0300 121.848)
- Trypsin/EDTA solution
- Dulbecco's modification of Eagle's liquid medium (DMEM, 4.5 g/l glucose, Invitrogen, cat. no. 52100-047) supplemented by 10% foetal calf serum (Invitrogen, cat. no. 10207-106)
- THOMA Chamber (Schütt Labortechnik cat. no. 9.161 080)

Methods

- Aspirate the medium from 35 mm tissue-culture plate containing ES-derived hepatic cells and rinse the cells 5 times with 3 ml of PBS one day before analysis.
- Incubate cells with HCM medium (without BSA and FCS) at 37°C for 24 hours.
- Collect supernatant into 1.5 ml tubes and store at –20°C for determination of albumin secretion.
- Add a sufficient amount of trypsin-EDTA solution to cover the whole surface and incubate at r.t. for 2-3 min.
- Remove carefully the trypsin-EDTA solution, add 3 ml of DMEM supplemented with 10% FCS.
- Resuspend the cells with a 5 ml glass pipette to obtain single cell suspension and determine the total cell number using THOMA chamber.
- Determine albumin content with ELISA according to manufacturer recommendations

5. MICROSCOPIC INSPECTION AND IMAGING OF SAMPLES LABELED BY IMMUNOFLUORESCENCE

Immuno-labelled preparations are examined by fluorescence microscopy. Where possible, we recommend the use of a confocal laser-scanning microscope (CLSM) instead of a conventional fluorescence microscope, as confocal images are superior. Furthermore, confocal microscopy allows the co-localization of proteins, which is especially relevant to the three-dimensional structures that are observed with, for example, ES-derived pancreatic cells. Figure 1 E shows a confocal image of an ES cell-derived, C-peptide-positive, islet-like cluster at stage 5+28d after induction of pancreatic differentiation; and Figure 1 F shows a confocal image of ES cell-derived, alpha-1-antitrypsin-positive cells at stage 5+39d after induction of hepatic differentiation.

6. Animal models

To test, whether the *in vitro* generated ES-derived progeny represent functional cells that may potentially be used to regenerate damaged or injured tissues, animal disease models with pathologic mechanisms and properties sufficiently similar to those of a specific human disease were developed. The animal diseases may be either induced or naturally occurring. For ES cell-derived pancreatic and hepatic cells, mouse and rat models of diabetes and liver diseases, respectively, have been established.

6.1 PANCREATIC ISLET REGENERATION MODELS

The non-obese diabetic (NOD) mouse model represents an inbred strain selected from outbred 'Swiss' mice in the course of breeding and selection experiments in Japan (25,26). In NOD mice and in a similar rat model ('BioBreeding', BB rats), insulin-producing beta cells are destroyed by an autoimmune process similar to the process occurring in patients with type 1-diabetes [insulin-dependent diabetes mellitus (IDDM) or juvenile diabetes [see (26)**].** Although, the understanding of pathogenetic mechanisms underlying type 1 diabetes in NOD mice is quite advanced, genus-specific differences that restrict their interpretation are unavoidable [for details, see (26)].

Destruction of pancreatic beta cells and the development of diabetes have been experimentally induced in laboratory animals by multiple low dose injections of streptozotocin (STZ) (2 7). The model has been successfully introduced for the treatment of hyperglycemia (as a consequence of beta cell destruction) by syngeneic islet transplantation (28). The dose of STZ is dependent on the animal strain and species used and has to be estimated before. Generally, concentrations in the range of 150 to 200 mg/kg body weight are

applied [for details, see (8-10,23,29)]; and cell suspensions ($1 \times 10^6 - 1 \times 10^7$) generated from stem cells are transplanted subcutaneously (9,29) into the renal subcapsular space (10,23,30,31) or into the spleen (8,10). The STZ model allows the use of appropriate mouse strains to minimize the risk of immune rejections after transplantation of cells into animals with different genetic background. However, a disadvantage of the STZ diabetes mouse model is the toxicity of streptozotocin to other organs, such as kidney and liver.

6.2 LIVER REPOPULATION MODELS

In contrast to most other parenchymal organs, such as kidney or pancreas, the liver is characterized by a very high organ regenerative capacity. Different cell types and mechanisms are involved in liver organ reconstitution depending on the type of liver injury, such as regeneration after partial hepatectomy, progenitor cell-dependent liver regeneration and liver repopulation by transplanted cells [rev. (32)]. ES cell-derived hepatic cells can be defined by their ability to repopulate the liver in animal models. For example, two transgenic mice models, the 'urokinase plasminogen activator (uPA) transgenic mice' and the 'fumarylacetoacetate (FAH) knockout mice' and a chemical liver injury rat model, the 'retrorsine model of rat, have been established.

Transgenic animals expressing the urokinase plasminogen activator (uPA) gene under control of the albumin promoter display liver inflammation with necrosis and a paucity of mature hepatocytes (33). The livers of albumin-uPA transgenic mice have been largely replaced by transplanted hepatocytes (34). In an extension of the model, the albumin-uPA transgene has been incorporated into immunotolerant (= immunodeficient uPA/recombinant activation gene-2, RAG-2) mice allowing the growth of hepatocytes from different species, including rat and human cells (35).

Another liver repopulation model represent fumarylacetoacetate knockout mice. The enzyme fumaryloacetoacetate hydrolase (FAH) catalyzes the final reaction in the tyrosine catabolic pathway. FAH deficiency results in accumulation of a hepatotoxic metabolite and is the cause of tyrosinema type I disease in human [HTI, (36)]. In this model, transplanted FAH-positive donor cells are used to repopulate FAH mutant liver (37). Because FAH deficiency is lethal in the neonatal period, the animals are treated with an inhibitor of tyrosine catabolism uspstream of FAH thus allowing the propagation of mutant animals and control of the selected pressure of transplanted cells [for details, see (38,39)]. Disadvantage of the transgenic mouse models is that liver nodules containing functional hepatocytes can develop spontaneously.

Another regeneration model is an approach based on liver repopulation in a chemical liver injury model in rat, the retrorsine-treated rats (40). After partial hepatectomy, the regenerative capacity of host cells is chemically blocked using lasiocarpine or retrorsine, substances structurally similar to pyrrolizidine alkaloids. These compounds are selectively metabolised to their active form by hepatocytes, where they alkylate cellular DNA and cause G2 cell cycle arrest (41). Disadvantage of this system is that after partial hepatectomy of retrorsine-treated animals, proliferation of endogenous, small, hepatocyte-like progenitor cells may be induced (42).

Due to the high regeneration capacity of the liver, in all liver disease models, the use of genetically labelled cells for transplantation is required. For all animal experiments, the guidelines of good animal experimental practice have to be followed.

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Figure legend

Figure 1. Morphology of pancreatic (A, C, E) and hepatic (B, D, F) cells differentiating from ES cells *in vitro* viewed by light (A-D), immunofluorescence (E) and confocal (F), microscopy (F). (A) Pancreatic progenitor cells 7 d after induction of differentiation (stage 5+16 d). (B) Hepatic progenitor cells 10 d after induction of differentiation (stage 5+19 d). Continued culture in HCM leads to the generation of further differentiated and specialized cell types. (C) Morphology of an islet-like cluster at stage 5+28 d. (D) Morphology of cuboidal hepatocyte-like (partially bi-nucleated) cells at stage 5+39 d. (E) Confocal image of an immuno-labelled, C-peptide-positive, islet-like cluster. (F) Confocal image of immuno-labelled alpha-1-antitrypsin-positive cells. Bars = 20 μm.

Tables

Table 1. Primer sequences, annealing temperature and expected sizes of amplified products for RT-PCR amplification of pancreas- and liver-specific genes

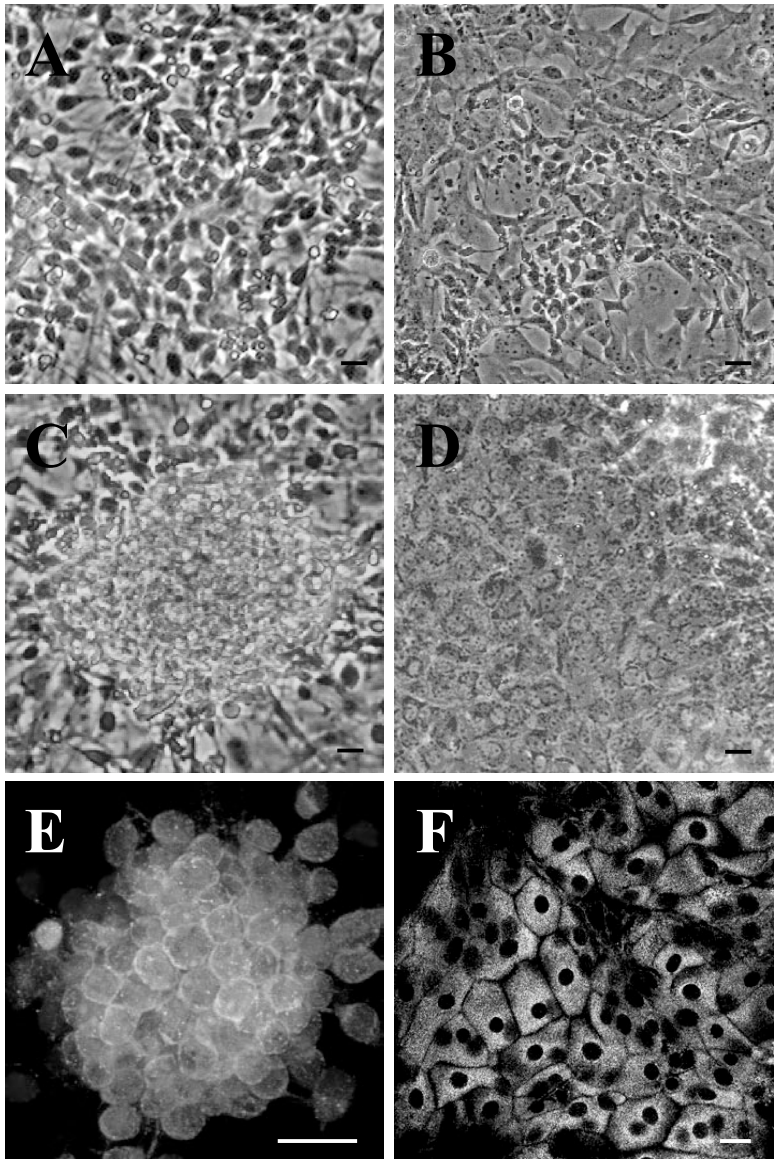
Gene	Primer sequence (Forward/Reverse)	Annealing temperature [°C]	Product size [bp]
Genes of pancreatic and hepatic progenitor cells			
Cytokeratin 19	5'-CTGCAGATGACTTCAGAACC 5'-GGCCATGATCTCATACTGAC	62	299
Nestin	5`-CTACCAGGAGCGCGTGGC 5`-TCCACAGCCAGCTGGAACTT	60	220
Pancreatic genes			
Glut-2	5'-TTCGGCTATGACATCGGTGTG 5'-AGCTGAGGCCAGCAATCTGAC	60	556
IAPP	5'-TGATATTGCTGCCTCGGACC 5'-GGAGGACTGGACCAAGGTTG	65	233
Insulin	5'-GTGGATGCGCTTCCTGCCCCTG 5'-ATGCTGGTGCAGCACTGA	64	288
Isl-1	5'-GTTTGTACGGGATCAAATGC 5'-ATGCTGCGTTTCTTGTCCTT	60	514
Ngn3	5'-TGGCGCCTCATCCCTTGGATG 5'-AGTCACCCACTTCTGCTTCG	60	157
Pax4	5'-ACCAGAGCTTGCACTGGACT 5'-CCCATTTCAGCTTCTCTTGC	60	300
Pax6	5'-TCACAGCGGAGTGAATCAG 5'-CCCAAGCAAAGATGGAAG	58	332
Pdx1	5'-CTTTCCCGTGGATGAAATCC 5'-GTCAAGTTCAACATCACTGCC	60	230
Hepatic genes			
Albumin	5'-GTCTTAGTGAGGTGGAGCAT 5'-ACTACAGCACTTGGTAACAT	58	569
Alpha-1-antitrypsin	5'-CAATGGCTCTTTGCTCAACA 5'-AGTGGACCTGGGCTAACCTT	63	518
Alpha-fetoprotein	5'-CACTGCTGCAACTCTTCGTA 5'-CTTTGGACCCTCTTCTGTGA	58	301
Cyp2b9	5'-GATGATGTTGGCTGTGATGC 5'-CTGGCCACCATGAAAGAGTT	53	153
Cyp2b13	5'-CTGCATCAGTGTATGGCATTTT 5'-TTTGCTGGAACTGAGACTACCA	65	166
HNF3 beta	5'-GCGGGTGCGGCCAGTAG 5'-GCTGTGGTGATGTTGCTGCTCG	63	378
Transthyretin	5'-CTCACCACAGATGAGAAG 5'-GGCTGAGTCTCTCAATTC	55	225
Tyrosine aminotransferase	5'-ACCTTCAATCCCATCCGA 5'-TCCCGACTGGATAGGTAG	50	206
Housekeeping gene			

Beta-tubulin	5’-TCACTGTGCCTGAACTTACC 5’-GGAACATAGCCGTAAACTGC	60	317

Table 2. Primary antibodies and fixation methods used for the detection of pancreatic and hepatic cells by immunofluorescence.

Protein	Antibody isotype	Working dilution	Supplier	Fixation	
				Met:Ac	PFA
Common pancreatic and hepatic proteins					
Cytokeratin 18	mouse IgG	1:100	Sigma	+	–
Cytokeratin 19	mouse IgM	1:100	Chemicon	+[1]	+[2]
Nestin	mouse IgG	1:3	Hybridoma Bank	+	+
Pancreatic proteins					
Carbonic anhydrase II	rabbit IgG	1:200	Abcam	–	+
C-peptide	guinea pig IgG	1:100	Linco	–	+
Insulin	mouse IgG	1:40	Sigma	–	+
Isl-1	rabbit IgG	1:200	Abcam	+	–
Hepatic proteins					
Albumin	sheep IgG	1:100	Serotec	+	–
Alpha-1-antitrypsin	rabbit IgG	1:100	Sigma	+	–
Alpha-fetoprotein	goat IgG	1:100	Santa Cruz	+	–
Amylase	goat IgG	1:100	Santa Cruz	+	–
Cytokeratin 14	mouse IgG	1:100	Sigma	+	–
Dipeptidyl peptidase IV	mouse IgG	1:100	Santa Cruz	+	–

[1]fixation method results in filament structures; [2]dot-like structures
Met: Ac – methanol: acetone solution
PFA – paraformaldehyde solution


A
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METHODS IN ENZYMOLOGY

Methods in Enzymology

Volume 365

Differentiation of Embryonic Stem Cells

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Table of Contents

hepatocytes. When these cells were cultivated as spheroids, these functions were maintained up to 4 months.[43]

As another direct induction method of hepatic lineage differentiation from ES cells, Geron corporation invented the method to differentiate human ES cells into hepatocyte-like cells by using chemical compounds (International patent application WO 01/81549 A2). In their protocol, human ES cells were cultured in the medium containing 20% serum replacement and 1% DMSO for the first 4 days. For the next 6 days, 2.5% sodium butyrate was added into the medium. Sixty-three percent of these cells were expressing albumin, and more than 80% of them were α1-antitrypsin positive by immunohistochemistry. None of them expressed α-fetoprotein. Cytochrome P450 activity was demonstrated in these cells for another indicator for *in vitro* liver function.

Conclusion and Future Directions

The potential to generate functional hepatoctyes *in vitro* from ES cells has been demonstrated by several different methods as described above. Embryoid body formation provides an appropriate microenvironment for hepatic differentiation. Hence, it may be a useful model to study liver development *in vitro*. Especially when combined with gene modulation at ES cell level, these methods may be useful to rapidly determine the role of genes in hepatic lineage commitment and/or hepatic functions.[32,44] It may not be ideal, however, to enrich and purify hepatic precursors from embryoid bodies because relative population of hepatocyte precursors is likely limited in embryoid bodies regardless of additional growth factors.

Several methods have been introduced to generate hepatic precursors more efficiently from ES cells without forming classical embryoid bodies as described above. Such methods may be more practical when we consider clinical applications of ES-derived hepatic precursors in the future. ES cell differentiation using a chemical agent, without introducing exogenous gene, would be particularly promising. Multiple studies have demonstrated that ES cell-derived hepatocytes function *in vivo*. It should be noted, however, none of them have yet shown restoration of liver disease models after transplantation of ES cell-derived hepatocytes. Other remaining challenges will be reconstructing liver *in vitro* as organoid using tissue engineering

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technique.[45] Continued work is necessary for a complete understanding of the potential of this promising cell population.

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[21] Differentiation of Mouse Embryonic Stem Cells into Pancreatic and Hepatic Cells

By GABRIELA KANIA, PRZEMYSLAW BLYSZCZUK, JAROSLAW CZYZ, ANNE NAVARRETE-SANTOS, and ANNA M. WOBUS

Introduction

Pancreas and liver cells are derivatives of the definitive endoderm. During embryogenesis, the pancreas develops from dorsal and ventral regions of the foregut,[1,2] whereas the liver originates from the foregut adjacent to the ventral pancreas compartment.[3] The development of pancreas and liver is regulated by specific transcription factors and signaling molecules. Pancreas development is controlled by signals from the notochord including TGF-β family members, activinβB and FGF-2, factors repressing sonic hedgehog (shh), a negative regulator of the homeobox gene Pdx1. The transcription factor Pdx1 is expressed in pancreatic buds and becomes restricted to β cells in the adult animal.[4,5] Islet-1 (Isl-1) and neurogenin3 (ngn3) regulate the development of early endocrine

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progenitors.[6,7] Two members of the Pax gene family, Pax6 and Pax4, are essential for the specification of endocrine α[8]- and β[9]-cells, respectively.

The first evidence of liver development is the expression of albumin and α-fetoprotein in endodermal cells.[10] Growth factors of the FGF family released from cardiac mesoderm activate endoderm to express liver- instead of pancreas-specific genes.[11] BMP signaling from the septum transversum mesenchyme is required for the hepatogenic response to FGF and for secondary inductions resulting in the outgrowth of liver buds.[12] BMP signaling maintains the endodermal expression of the transcription factor GATA-4,[13] which together with HNF3β, is essential for the hepatic specification of endoderm.[14,15] Additional signaling molecules, such as hepatocyte-growth factor (HGF),[16] c-met,[17] c-jun[18] and β1-integrins[15] are involved in liver development. There is evidence that albumin-expressing cells from the endoderm (hepatoblasts) differentiate into hepatocytes and bile duct cells.[3]

Due to the high incidence of liver- and pancreas-related diseases in the human population, and the lack of suitable donor cells and tissues for transplantation, the need of generating a "surrogate" pancreatic and hepatic cell population has been strengthened. Several sources of cells for transplantation are considered including fetal and adult stem cells, or genetically modified cell lines. An alternative source for generating transplantable cells are embryonic stem (ES) cells which have an almost unlimited proliferation capacity, while retaining the potential to differentiate *in vitro* into cells of all

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three primary germ layers.[19,20] It has been shown, that ES cells can be successfully differentiated into insulin-producing cells of mouse[21,22] and human[23] origin, as well as into functional hepatocyte-like cells *in vitro*.[24–27]

The close relationship between pancreas and liver development is substantiated by the findings that ductal epithelial pancreatic progenitor cells may be reprogrammed into liver cells[28] and, vice versa, liver stem cells may be directed into the pancreatic endocrine lineage.[29] Therefore, it was postulated that pancreatic and hepatic progenitor cells share a common stem/progenitor cell population.[30] Cells expressing the intermediate filament protein nestin may represent such a progenitor cell type, because *in vitro*, nestin-positive (nestin+) cells selected from ES cells have been differentiated into pancreatic endocrine[22,31–33] and hepatic[31] cells after addition of specific growth and extracellular matrix factors. *In vivo*,

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nestin is transiently expressed in different cell types of embryonic and adult tissues, including the developing central nervous system,[34,35] skeletal muscle,[36] heart,[37] endothelial,[38] mesenchymal pancreatic,[39,40] and hepatic stellate[41] cells and is suggested to play a transient role in various proliferation and migration processes of progenitor cells.[42] Although, nestin+ cells may participate in the neogenesis of endocrine islet cells,[31,43] recent data showed that *in vivo*, nestin is expressed in mesenchymal and not in epithelial cells, where endocrine progenitors reside.[39] We therefore, speculate that nestin+ cells are characterized by a high developmental plasticity and represent a progenitor cell population which *in vitro* under the influence of genetic[33] or epigenetic factors[22] can be programmed into pancreatic endocrine[22,31] or into hepatic[29] cells. As known for many years, nestin+ cells also develop efficiently into neural[35] cell fates *in vitro*.

Here, we describe methods to direct ES cells into pancreatic and hepatic cells via the generation of nestin+ cells. In comparison to the normal ("basic") differentiation protocol,[20] selection of nestin+ cells resulted in qualitatively similar cell types, but the "nestin+ cell selection" protocol was much more efficient to generate insulin-[33] and albumin-producing cells. In addition, a histotypic differentiation model is presented. It is known for a long time that the three-dimensional organization of cells *in vitro* results in increased tissue-specific functions of various cell types.[44–48] The histotypic

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[48] J. R. Friend, F. J. Wu, L. K. Hansen, R. P. Remmel, and W.-S. Hu, *in* "Tissue Engineering Methods and Protocols" (J. R. Morgan and M. L. Yarmush, eds.), Methods in Molecular Medicine, vol. 18: pp. 245–252. Humana Press Inc., Totowa, NJ, 1999.

culture model enabled the generation of highly differentiated islet- and hepatocyte-like spheroids.

Material and Methods

Culture of Undifferentiated ES Cells

Embryonic stem (ES) cells of line R1[49] were cultivated on a feeder layer of primary mouse embryonic fibroblasts on gelatin (0.1%)-coated petri dishes (Falcon Becton Dickinson, Heidelberg, Germany) in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Life Technologies, Eggenstein, Germany) supplemented with 15% heat-inactivated fetal calf serum (FCS, selected batches, Gibco), L-glutamine (Gibco, 2 m*M*), β-mercaptoethanol (Serva, Heidelberg, Germany, final concentration 5×10^{-5} *M*), nonessential amino acids (Gibco, stock solution diluted 1 : 100), penicillin–streptomycin (Gibco, stock solution diluted 1 : 100) and 10 ng/ml recombinant human leukemia inhibitory factor (LIF, for preparation, see Ref. 50) as described.[20]

Differentiation of ES cells into Pancreatic and Hepatic Cells

Two protocols were comparatively investigated, the "basic" protocol and the "nestin+ cell selection" protocol:

1. According to the "basic" protocol, ES cells were differentiated as embryoid bodies (EB) via the "hanging drop" method[20] and after plating, the cells were cultured in the specific differentiation media. This protocol generates a population of different cell types including pancreatic[33] and hepatic cells.
2. By the "nestin+ cell selection" protocol, ES cells were cultured as EB and after plating, nestin+ progenitor cells were selected in growth factor-containing, but serum-free medium. Further, the nestin+ cells were directed either into pancreatic or hepatic (or neural) cell types by specific differentiation factors. Whereas both protocols, in principle, gave rise to pancreatic and hepatic cell types, the "nestin+ cell selection" protocol resulted in higher differentiation efficiency and an increased number of mature cells compared to the "basic" protocol.[33]

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[50] J. Rohwedel, U. Sehlmeyer, J. Shan, A. Meister, and A. M. Wobus, *Cell Biol. Int.* **20**, 579–587 (1996).

Selection of Nestin+ Cells

ES cells ($n = 200$) were cultivated as EBs in "hanging drops" for two days and after transfer into bacteriological plates (Greiner, Germany) for 2 days in suspension in Iscove's modification of DMEM (IMDM, Gibco) supplemented with 15% FCS, L-glutamine, nonessential amino acids (see above) and α-monothioglycerol (Sigma, Steinheim, Germany; final concentration 450 μM) instead of β-mercaptoethanol. Penicillin–streptomycin may be added to the cultures (see above). At day 4, EBs ($n = 30$) were plated onto gelatin-coated 6 cm tissue culture dishes (Nunc, Germany) and cultivated in IMDM supplemented by 10% FCS. The optimal density of EB-derived nestin+ cells is critical for the efficient generation of pancreatic and hepatic cell types. At day 4+1, the medium was exchanged by DMEM/F12 (Gibco) supplemented with 5 μg/ml insulin, 30 nM sodium selenite (both from Sigma), 50 μg/ml transferrin, 5 μg/ml fibronectin (both from Gibco) without FCS and cells were cultivated for further 7 days.[51]

ES Cell Differentiation into Pancreatic or Hepatic Cells

Differentiation into Pancreatic Cells

At day 4+8, EB outgrowths were dissociated by 0.1% trypsin (Gibco)/0.08% EDTA (Sigma) in PBS (1 : 1) for 1 min, collected by centrifugation and replated onto tissue culture plates (Nunc) in DMEM/F12 containing 20 nM progesterone, 100 μM putrescine, 1 μg/ml laminin, 10 mM nicotinamide (all from Sigma), B27 media supplement (Gibco), 25 μg/ml insulin, 50 μg/ml transferrin, 5 μg/ml fibronectin, and 30 nM sodium selenite (="pancreatic differentiation medium") supplemented with 10% FCS and penicillin–streptomycin (see above). For immunofluorescence analysis, cells were plated onto poly-L-ornithine/laminin-coated cover slips, onto 3 cm culture dishes (ELISA), and onto 6 cm culture dishes for RT-PCR and further histotypic culture. At Day 4+9, FCS was removed and the cells were cultivated until day 4+8+20 or other stages (Fig. 1A).[33]

Differentiation into Hepatic Cell Types

At Day 4+8, EBs were dissociated by 0.1% trypsin (Gibco)/0.08% EDTA (Sigma) in PBS (1 : 1) for 1 min, collected by centrifugation and replated in Hepatocyte Culture Medium (HCM, "hepatic differentiation medium"). HCM composed of 500 ml Hepatocyte Basal Medium (Modified Williams' E), 0.5 ml ascorbic acid, 10 ml BSA-FAF

[51] A. Rolletschek, H. Chang, K. Guan, J. Czyz, M. Meyer, and A. M. Wobus, *Mech. Dev.* **105**, 93–104 (2001).

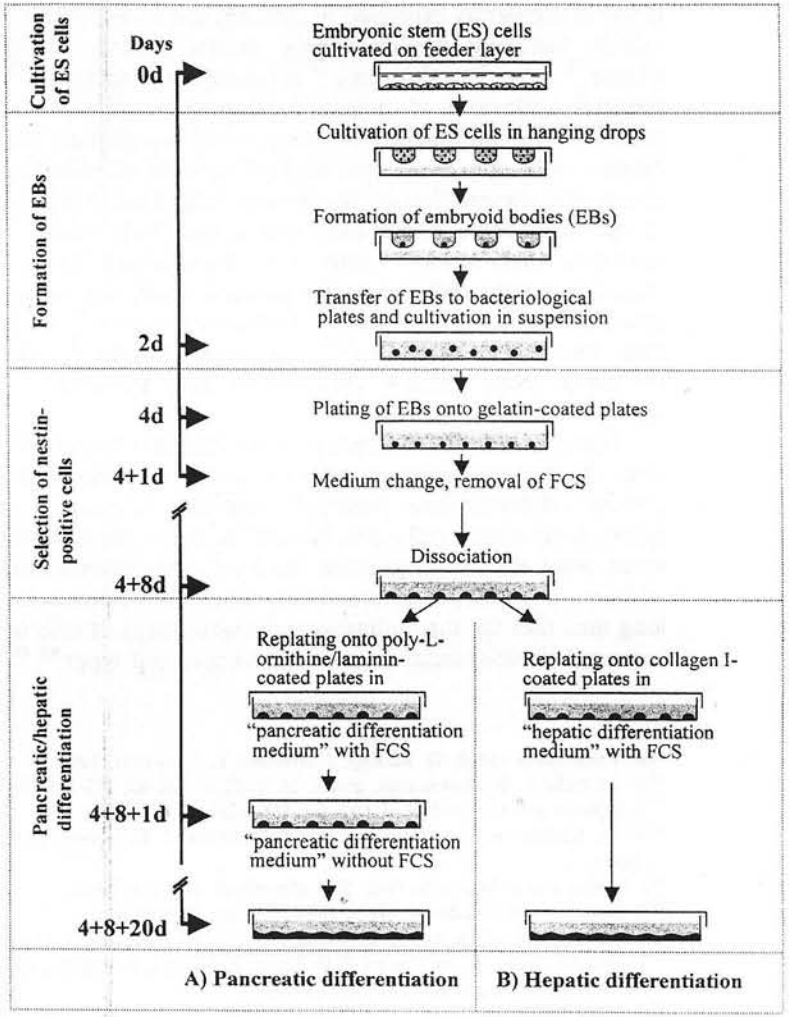

FIG. 1. Schematic presentation of the "nestin+ cell selection" protocol used to differentiate ES cells via embryoid bodies (EBs) and differentiation induction into pancreatic (A) and hepatic (B) cell types.

(bovine serum albumin-fatty acid free), 0.5 ml hydrocortisone, 0.5 ml transferrin, 0.5 ml insulin, 0.5 ml human epithelial growth factor (EGF) and 0.5 ml gentamycin-amphothericin (GA-1000; all from Clonetics, Bio-Whittaker; A Cambrex Company; Belgium) is supplemented by 20% FCS and penicillin–streptomycin (see above) and prepared

immediately before use. For immunofluorescence, cells were transferred onto collagen type I-coated cover slips, onto 3 cm culture dishes (RT-PCR, ELISA) and onto 6 cm collagen type I-coated plates for further histotypic culture. The cells were cultivated until day 4+8+20 or other stages (Fig. 1B).

Histotypic Differentiation into Spheroids

The three-dimensional histotypic culture system was adapted for the differentiation and maturation of pancreatic and hepatic cells generated by the "nestin+ cell selection" protocol. During cultivation in "spinner culture" as spheroids, the cells aggregate, proliferate and establish specific cell-to-cell interactions. A comparison of spheroids with monolayer cultures showed that spheroids resulted in higher proliferation rates, and their differentiation properties closely resembled the *in vivo* situation.[44]

ES-derived cells cultivated according to the "nestin+ cell selection" protocol for 4+8+20 (pancreatic cells) or 4+8+14 days (hepatic cells), were dissociated for 1 min by 0.1% trypsin: 0.08% EDTA (1:1) in PBS (pancreatic cells) or 0.2% trypsin/0.02% EDTA (1:1) in PBS (hepatic cells), respectively.

Cells ($n = 1.5–2 \times 10^7$) were collected by centrifugation and transferred into 6 cm bacteriological petri dishes in "pancreatic" or "hepatic differentiation medium" (note that "hepatic differentiation medium" is supplemented by 10% FCS).

After 24 hr of static suspension culture, cell aggregates were transferred into 100 ml "spinner" culture flasks and cultivated in "pancreatic" or "hepatic differentiation medium" ("hepatic differentiation medium" supplemented by 10% FCS) by the CELLSPIN system (Cellspin, Integra Bioscience AG, Switzerland) at 30 rpm agitation at 37°C up to 10 days.

Analysis of Differentiated Phenotypes

The methods presented here allow the qualitative and quantitative determination of pancreas- and liver-specific markers at the mRNA (RT-PCR) and protein (immunofluorescence, immunohistochemistry) level. In addition, functional properties are detected by ELISA.

Semi-Quantitative RT-PCR Analysis

ES-derived cells were collected and suspended in lysis buffer composed of 4 *M* guanidinium thiocyanate, 25 m*M* sodium citrate (pH 7);

0.5% (w/v) sarcosyl and 0.1 *M* β-mercaptoethanol. Total RNA was isolated by the single step extraction method according to Chomczynski and Sacchi.[52] mRNA was reverse transcribed using PolyT tail primer Oligo d(T) and MuLV reverse transcriptase (Perkin Elmer, Überlingen, Germany).

cDNAs were amplified using oligonucleotide primers complementary to transcripts of the analyzed genes (see Table I) and AmpliTaq DNA polymerase (Perkin Elmer) as described.[20,53] The PCR reaction was electrophoretically separated on 2% (w/v) agarose gels containing 0.35 μg/ml of ethidium bromide and gels were illuminated with UV light, stored by E.A.S.Y. system (Herolab GmbH, Wiesloch, Germany) and analyzed by the TINA2.08e software (Raytest Isotopenmeßgeräte GmbH, Straubenhardt, Germany).

The intensity of the ethidium bromide fluorescence signals was determined from the area under the curve for each peak and the data of target genes were plotted as percentage changes in relation to the expression of the housekeeping gene β-tubulin. Expression of β-tubulin served to normalize the amounts of RT-products of all samples. The sample of the target gene with the highest ratio of the gene product in relation to β-tubulin was set as 100% and served as reference for the other samples of the target gene.[20]

Immunofluorescence Analysis

For immunofluorescence, EB outgrowths of ES cells growing on cover slips were either fixed with 4% paraformaldehyde (PFA) in PBS at room temperature (RT) for 20 min or in methanol:acetone (Met:Ac; 7:3) at −20°C for 10 min, depending on the antibody used (see Table II). After rinsing (3×) in PBS, bovine serum albumin (BSA, 1% in PBS) was used to inhibit unspecific labeling (30 min) at RT. Cells were incubated with the primary antibodies in specific dilutions (Table II) at 37°C for 60 min. Samples were washed (3×) in PBS and incubated with fluorescence-labeled secondary antibodies (diluted in 1% BSA in PBS) at 37°C for 60 min (Table III). To label the nuclei for a semi-quantitative estimation of immunofluorescence signals, cells were incubated in 5 μg/ml Hoechst 33342 in PBS at 37°C for 10 min. After washing (3×) in PBS and (1×) in Aqua dest., the specimens were embedded in mounting medium (Vectashield, Vector Laboratories Inc., Burlingame, CA, USA). Labeled cells were analyzed by the fluorescence microscope ECLIPSE TE300 (Nikon, Japan), or the

[52] P. Chomczynski and N. Sacchi, *Anal. Biochem.* **162**, 156–159 (1987).
[53] A. M. Wobus, G. Kaomei, J. Shan, M. C. Wellner, J. Rohwedel, G. Ji, B. Fleischmann, H. A. Katus, J. Hescheler, and W. M. Franz, *J. Mol. Cell Cardiol.* **29**, 1525–1539 (1997).

TABLE I
PRIMERS FOR RT-PCR ANALYSIS

Gene	Primer sequence (Forward/Reverse)	Annealing temperature	No. of cycles	Product size
Pancreatic markers:				
Glut-2	5′-TTCGGCTATGACATCGGTGTG 5′-AGCTGAGGCCAGCAATCTGAC	60°C	40	556 bp
IAPP	5′-TGATATTGCTGCCTCGGACC 5′-GGAGGACTGGACCAAGGTTG	65°C	40	233 bp
Insulin	5′-CCCTGCTGGCCCTGCTCTT 5′-ATGCTGGTGCAGCACTGA	60°C	40	270 bp
Isl-1	5′-GTTTGTACGGGATCAAATGC 5′-ATGCTGCGTTTCTTGTCCTT	60°C	35	514 bp
Ngn3	5′-TGGCGCCTCATCCCTTGGATG 5′-AGTCACCCACTTCTGCTTCG	60°C	40	157 bp
Pax4	5′-ACCAGAGCTTGCACTGGACT 5′-CCCATTTCAGCTTCTCTTGC	60°C	40	300 bp
Pax6	5′-TCACAGCGGAGTGAATCAG 5′-CCCAAGCAAAGATGGAAG	58°C	35	332 bp
Pdx1	5′-CTTTCCCGTGGATGAAATCC 5′-GTCAAGTTCAACATCACTGCC	60°C	45	230 bp
Shh	5′-TGAGGACGGCCATCATTCAG 5′-CTCCAGCGTCTCGATCACGT	59°C	45	173 bp
Hepatic markers:				
albumin	5′-GTCTTAGTGAGGTGGAGCAT 5′-ACTACAGCACTTGGTAACAT	58°C	35	569 bp
α-1-antitrypsin	5′-CAATGGCTCTTTGCTCAACA 5′-AGTGGACCTGGGCTAACCTT	63°C	30	518 bp
α-fetoprotein	5′-CACTGCTGCAACTCTTCGTA 5′-CTTTGGACCCTCTTCTGTGA	58°C	35	301 bp
HNF3β	5′-GCGGGTGCGGCCAGTAG 5′-GCTGTGGTGATGTTGCTGCTCG	63°C	40	378 bp
Housekeeping gene:				
β-tubulin	5′-TCACTGTGCCTGAACTTACC 5′-GGAACATAGCCGTAAACTGC	60°C	28	317 bp

confocal laser scanning microscope (CLSM) LSM-410 (Carl Zeiss, Jena, Germany) using the following excitation lines/barrier filters: 364 nm/450-490BP (Hoechst 33342), 488 nm/510-525BP (FITC/DTAF), 543 nm/570LP (Cy3).

Semi-Quantitative Determination of Immunofluorescence Signals

Quantification of immunofluorescence signals was performed by two alternative methods depending on the cell culture status. Cells growing in

TABLE II
SELECTED PRIMARY ANTIBODIES TO CHARACTERIZE NESTIN+ PROGENITOR CELLS, PANCREATIC AND HEPATIC CELL TYPES

Primary antibody	Dilution	Company	Fixation
Progenitor cells:			
mouse anti-nestin (clone rat 401)	1:3	Development Studies Hybridoma Bank, IA, USA	4% PFA
Pancreatic markers:			
mouse anti-insulin	1:40	Sigma	4% PFA
rabbit anti-glucagon	1:40	Dako Corporation, CA, USA	4% PFA
rabbit anti-somatostatin	1:40	Dako	4% PFA
rabbit anti-PP	1:40	Dako	4% PFA
Hepatic markers:			
goat anti-α-fetoprotein	1:100	Santa Cruz Biotechnol, USA	Met:Ac (7:3)
goat anti-amylase	1:100	Santa Cruz	Met:Ac (7:3)
goat anti-dipeptidyl peptidase IV	1:100	Santa Cruz	Met:Ac (7:3)
sheep anti-albumin	1:100	Serotec, USA	Met:Ac (7:3)
rabbit anti-α1-antitrypsin	1:100	Sigma	Met:Ac (7:3)
mouse anti-cytokeratin 18 (clone KS-B17.2)	1:100	Sigma	Met:Ac (7:3)
mouse anti-cytokeratin 14 (clone CKB1)	1:100	Sigma	Met:Ac (7:3)
mouse anti-cytokeratin 19	1:100	Chemicon, Hofheim, Germany	Met:Ac (7:3)
mouse anti-glutamine synthetase	1:100	BD Transduction Laboratories, USA	Met:Ac (7:3)

TABLE III
FLUORESCENCE-LABELED SECONDARY ANTIBODIES USED FOR IMMUNOFLUORESCENCE ANALYSIS

Secondary antibody	Dilution	Company
Cy^{3TM}-conjugated goat anti-mouse IgG	1:600	
Cy^{3TM}-conjugated goat anti-rabbit IgG	1:600	Jackson
Cy^{3TM}-conjugated donkey anti-goat IgG	1:600	ImmunoResearch
Cy^{3TM}-conjugated goat anti-mouse IgM	1:600	Laboratories,
DTAF-conjugated goat anti-rabbit IgG	1:100	Dianova, Hamburg,
FITC-conjugated donkey anti-sheep IgG	1:100	Germany
FITC-conjugated goat anti-mouse IgG	1:100	

monolayer may be analyzed by direct determination of immunolabeled cells (percentage values), whereas, for cells growing in multilayered clusters, the "labeling index" technique is proposed.

1. *Determination of percentage values of Hoechst-labeled cells:* Cells were analyzed for immunofluorescence signals and the percentage number of immuno-positive cells relative to a total number of ($n = 1000$) Hoechst 33342-labeled cells is given.
2. *Estimation of the "labeling index":*[33] For cells growing in clusters, immunofluorescence analysis was performed using the inverted fluorescence microscope ECLIPSE TE300 (Nikon, Japan) equipped with a 3CCD Color Video Camera DXC-9100P (Sony, Japan) and the LUCIA M-Version 3.52a software (LIM, Czech Rep.) For each sample, at least 20 randomly, but representative selected pictures were analyzed for the "area fraction" value, which is the ratio of the immunopositive signal area to the measured area. To discriminate the immunopositive signal from background fluorescence, the pictures were binarized with the specific threshold fluorescence values.

Immunohistochemistry (IHC) of Spheroids

Spheroids were collected by sedimentation, washed two times with PBS, fixed in Bouin's solution (75 ml picric acid, 25 ml 4% formaldehyde, 5 ml acetic acid) for 2 hr at RT, washed twice in 70% ethanol and dehydrated in graded ethanol. Fixed spheroids were embedded in paraffin, sectioned at 5 μm slices and mounted on silanized slides using conventional techniques. Slides were deparaffinized at 60°C for 2 hr, rehydrated in xylene (5 min, RT), isopropanol (5 min, RT), processed through graded ethanol (96, 80, 70 and 50%, 5 min each, RT), washed in Aqua dest. (1×, 3 min, RT) and in PBS (3×, 5 min, RT). Immunohistochemistry was performed as described in section "Immunofluorescence analysis" beginning with BSA blocking.

Insulin ELISA

ES-derived cells differentiated into the pancreatic lineage were washed PBS (5×) and preincubated in freshly prepared KRBH (Krebs' Ringer Bicarbonate Hepes) buffer containing 118 m*M* sodium chloride, 4.7 m*M* potassium chloride, 1.1 m*M* potassium dihydrogen phosphate, 25 m*M* sodium hydrogen carbonate (all from Carl Roth GmbH & Co, Karlsruhe, Germany), 3.4 m*M* calcium chloride (Sigma), 2.5 m*M* magnesium sulfate (Merck), 10 m*M* Hepes and 2 mg/ml bovine serum albumin supplemented with 2.5 m*M* glucose (all from Gibco) for 90 min at 37°C.

To estimate glucose-induced insulin secretion, the buffer was replaced by 27.7 m*M* glucose and alternatively with 5.5 m*M* glucose and 10 μM tolbutamide dissolved in KRBH buffer for 15 min at 37°C. The control was incubated in KRBH buffer supplemented with 5.5 m*M* glucose. The supernatant was collected and stored at −20°C for determination of insulin release.

Cells were dissociated by 0.2% trypsin: 0.02% EDTA in PBS (1 : 1) for 3 min and collected by centrifugation. Proteins were extracted from the cells with acid ethanol (1 *M* hydrochloric acid : absolute ethanol 1 : 9) and overnight incubation at 4°C, followed by cell sonification, and stored at −20°C for the determination of total cellular insulin and protein content, respectively. The insulin enzyme-linked immunosorbent assay (ELISA, Mercodia AB, Sweden) was performed according to manufacturer recommendations.

The total protein content was determined by the protein Bradford assay according to manufacturer recommendations (Bio-Rad Laboratories GmbH, Munchen, Germany). Released insulin levels are presented as ratio of released insulin per 15 min and intracellular insulin content. The intracellular insulin level is given as ng insulin per mg protein (see Ref. 33).

Albumin ELISA

ES-derived cells were washed in PBS (5×) and incubated at 37°C in "hepatic differentiation medium" in the absence of BSA/FCS for 24 hr. The supernatant was collected and stored at −20°C for measurement of albumin release.

To determine the total cell number, cells were dissociated by treatment with 0.2% trypsin: 0.02% EDTA (1 : 1) in PBS.

For the estimation of albumin synthesis, the quantitative enzyme-linked immunoassay (Albumin ELISA, Bethyl Laboratories, INC, Montgomery, USA) was performed according to manufacturer recommendations.

Results

By applying the "nestin+ cell selection" protocol followed by specific differentiation induction in the presence of specific differentiation factors, insulin- (Fig. 1A) and albumin- (Fig. 1B) producing cells were generated at high efficiency.[33]

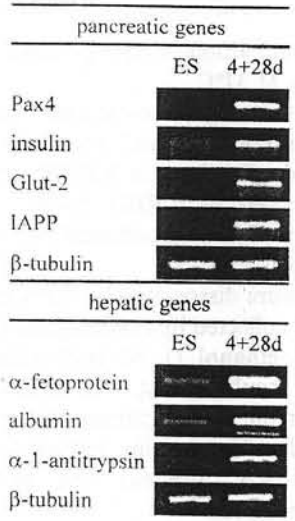

FIG. 2. RT-PCR analysis of pancreatic [Pax4, insulin, glucose transporter-2 (Glut-2), islet amyloid polypeptide (IAPP)] and hepatic [albumin, α-fetoprotein, α-1-antitrypsin] genes. mRNA levels of target genes in undifferentiated ES cells and in cells cultivated according to the "nestin+ cell selection" protocol and differentiated into pancreatic and hepatic cell types were analyzed at stage 4+28 d (=4+8+20 d). The housekeeping gene β-tubulin was used as internal standard.

Differentiation of Pancreatic Cells

During differentiation into the pancreatic lineage, genes specifically expressed in β cells including Pax4, insulin, glucose transporter-2 (Glut-2) and islet amyloid polypeptide (IAPP) were significantly upregulated at stage 4+28 d (=4+8+20 d) in comparison to undifferentiated ES cells (Fig. 2). The number of pancreatic endocrine cells amounted to a "labeling index" of 0.15 (insulin) and 0.05 (glucagon), respectively, corresponding to about 20–25% insulin- and 5–10% glucagon-producing cells (Fig. 3A). The number of insulin-positive cells increased with differentiation time from Day 4+8+13 to Day 4+8+20, whereas no significant changes were detected in the number of glucagon-expressing cells (Fig. 3A). Intracellular insulin levels analyzed by ELISA amounted to 20.7 ng insulin/mg protein at stage 4+8+20 d.[33] The cells released insulin in response to glucose.[33]

Cells generated according to the "nestin+ cell selection" protocol for 4+8+20 days followed by 10 days of histotypic "Spinner" culture showed a significant accumulation of insulin in spheroids (Fig. 4B). The intracellular insulin levels amounted to 297.4±18.0 ng/mg protein.[33]

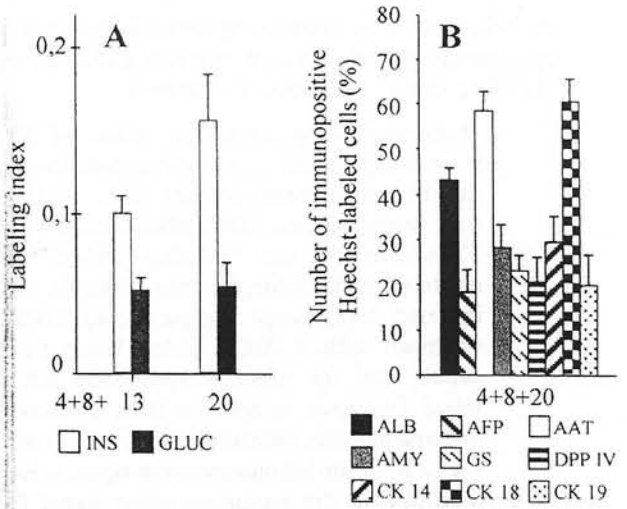

FIG. 3. Semi-quantitative imaging analysis of pancreatic and hepatic proteins in ES-derived cells analyzed for the "labeling index" at days 4+8+13 and 4+8+20 (A), and the determination of percentage values of Hoechst 33342-labeled cells expressing hepatic proteins (B) at stage 4+8+20 d. Each value represents mean±SEM. Abbreviations: INS, insulin; GLUC, glucagon; ALB, albumin; AFP, α-fetoprotein; AAT, α-1-antitrypsin; AMY, amylase; GS, glutamine synthetase; DPP IV, dipeptidyl peptidase IV; CK 14, cytokeratin 14; CK 18, cytokeratin 18; CK 19, cytokeratin 19. (A) $n=3$ experiments with 2 parallels, (B) $n=13$ experiments.

Differentiation of Hepatic Cells

After differentiation of ES cells via the "nestin+ cell selection" protocol and differentiation induction into the hepatic lineage (Fig. 1B), liver-specific genes encoding α-fetoprotein, albumin and α-1-antitrypsin were significantly upregulated at stage 4+28 d (=4+8+20 d) in comparison to undifferentiated ES cells (Fig. 2). Liver-specific proteins were efficiently expressed as determined by immunofluorescence analysis. Albumin (ALB) was produced in up to 45%, α-fetoprotein (AFP) in about 20%, α-1-antitrypsin (AAT) and cytokeratin 18 (CK 18) in nearly 60% of Hoechst 33342-labeled cells. Amylase (AMY), glutamine synthetase (GS), dipeptidyl peptidase IV (DPP IV), cytokeratin 14 (CK 14) and cytokeratin 19 (CK 19) were detected in about 20 to 30% of the total number of Hoechst 33342-labeled cells (Fig. 3B). All these proteins were determined *in vivo* to be specific for the different hepatic phenotypes including hepatocytes, biliary duct epithelium, hepatoblasts and oval cells (for review see Ref. 15). Cells generated according to the "nestin+ cell selection" protocol for 4+8+14 days followed by 10 d of histotypic "Spinner" cultivation showed a significant accumulation of albumin (Fig. 4C) in spheroids.

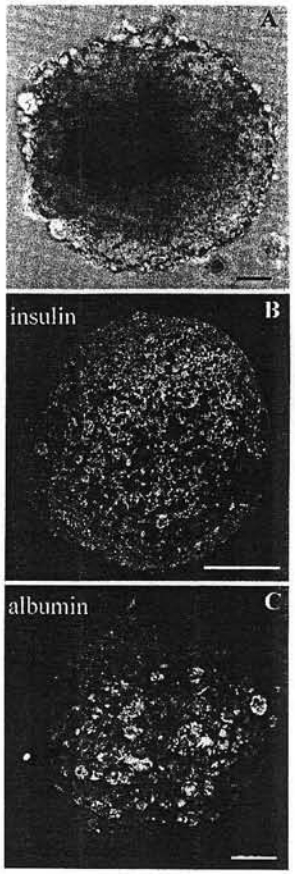

FIG. 4. Phase contrast (A) and immunofluorescence micrographs of insulin (B)- and albumin (C)-labeled cells (white) in ES-derived spheroids generated via the "nestin+ cell selection" protocol, specific differentiation induction and 10 days maturation in the "spinner" culture system. Bar = 50 μm.

Summary

Here, we present efficient strategies to differentiate ES cells either into pancreatic or into hepatic cell types. We recommend a strategy to select nestin+ cells, an early progenitor cell type with high developmental plasticity, followed by differentiation induction with specific growth and extracellular matrix factors into pancreatic and hepatic cell types. Cells differentiating via nestin+ cells into the pancreatic and hepatic lineage expressed tissue-specific genes. Proteins characteristic for mature endocrine pancreatic or hepatic cells were synthesized and released. Further, a histotypic "spinner" culture system was introduced to generate mature insulin- and albumin-producing cells at high efficiency.

Acknowledgments

We want to thank Mrs. S. Sommerfeld, O. Weiß and K. Meier for excellent technical assistance. The financial support by the Deutsche Forschungsgemeinschaft (DFG, WO 503/3-2), DeveloGen AG, Göttingen and Fonds der Chemischen Industrie (FCI), Germany, is gratefully acknowledged.

[22] Generating CNS Neurons from Embryonic, Fetal, and Adult Stem Cells

By JONG-HOON KIM, DAVID PANCHISION, RAJA KITTAPPA, and RON MCKAY

Introduction

In vitro culturing of neuroepithelial stem cells has become an indispensable tool for studying the mechanisms controlling proliferation, mitotic arrest and lineage commitment of cells in the nervous system.[1] Recent progress in this field has improved the possibility of treating patients by transplanting new cells that can replace those lost through trauma or disease. However, any potential clinical use of these cells requires systematic methods to enrich for the cell of interest and demonstrate that these cells show functions that will assist in understanding and treating the disease.

Embryonic stem (ES) cells are pluripotent and immortal cells derived from the inner cell mass of preimplantation blastocysts. They can proliferate extensively and have ability to differentiate into endodermal, mesodermal and ectodermal derivatives. The most important benefit of using ES cells as donor cells is the relative ease of genetic engineering, which permits the enrichment or purification for specific cell types by selectable marker. Recently, we have developed efficient methods of generating central nervous system (CNS) stem cells and their derivatives from ES cells.[2–5]

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Embryonic stem cell-derived cardiac, neuronal and pancreatic cells as model systems to study toxicological effects

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Abstract

Primary cultures or established cell lines of vertebrates are commonly used to analyse the cytotoxic potential of chemical factors, drugs and xenobiotics in vitro. An alternative approach will be provided by permanent lines of pluripotent embryonic stem (ES) cells, which are able to differentiate into specialised somatic cell types in vitro. Here, we demonstrate the capacity of ES cells to generate functional cardiac, neuronal and pancreatic cells. We show that during ES cell differentiation, tissue-specific genes, proteins as well as functional properties are expressed in a developmentally regulated manner recapitulating processes of early embryonic development. We present data that show the use of ES-derived cardiomyocytes and dopaminergic neurons in toxicological studies and the potential of ES-derived pancreatic beta-like cells in future in vitro assays. The application of these differentiation systems to human ES cells opens up new perspectives in basic and applied toxicology.

Keywords: Embryonic stem cells; Differentiation; Cytotoxicity; Embryotoxicity; Cardiac cells; Dopaminergic neurons; Beta-cells

1. Introduction

Cell culture systems are well established as cellular screening models in toxicology. However in many cases, the in vitro models, such as primary cultures or established cell lines do not represent the functional properties of specialised somatic cells. In vitro culture often results in a loss of proliferation capacity, viability and tissue-specific properties during long-term cultivation (Chamley et al., 1977; see Wobus et al., 1994a; Gottlieb, 2002) and tissue-specific characteristics may be impaired in established lines of cardiac, neuronal or pancreatic cells (see Greene and Tischler, 1976; Wobus et al., 1994a; Brismar, 1995; Murayama et al., 2001).

An alternative approach to generate functional cell types in vitro has been offered by the establishment of embryonic stem (ES) cells, pluripotent stem cells cultured from early embryos of mouse (Evans and Kaufman, 1981; Martin, 1981; Wobus et al., 1984) and human (Thomson et al., 1998). ES cells are undifferentiated cells with the capacity to develop into cells of all three primary germ layers, and after differentiation in vitro give rise to numerous specialised cell types of the body, including cardiac, skeletal and vascular smooth muscle, neuronal, glial, epithelial, hematopoietic, chondrogenic, osteogenic (rev. Rohwedel et al., 2001), pancreatic (Soria et al., 2000; Blyszczuk et al., 2003), hepatic (Jones et al., 2002; Kania et al., 2003) and germ (Hübner et al., 2003) cells. All these studies

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showed that during in vitro differentiation, ES cells recapitulate cellular developmental processes and gene expression patterns of early embryogenesis and result in functionally competent specialised cell types (rev. Guan et al., 2001; Rohwedel et al., 2001).

During the last years, ES cell-derived phenotypes have been used in several applications including pharmacological, cytotoxicological and embryotoxicological approaches (="ES cell technology", Rohwedel et al., 2001; rev. Wobus, 2001), and transplantation experiments showed the functional capacity of ES-derived cell types (rev. Wobus, 2001).

The recent isolation and generation of human ES cells opens future possibilities for such studies by the use of human cellular derivatives including cardiac, neuronal or pancreatic cells (rev. Schuldiner et al., 2000; Lebkowski et al., 2001; NIH Report, 2001).

The article presents a short overview on the properties of ES-derived cardiac, neuronal and pancreatic cells and their potential use in basic and applied toxicology.

2. Material and methods

2.1. ES cell culture and differentiation

ES cells of lines R1 (Nagy et al., 1993) and D3 (Doetschman et al., 1985) cultivated on a feeder layer of primary mouse embryonic fibroblasts were used (Wobus et al., 2002). For differentiation, cells were cultivated in three-dimensional aggregates or "embryoid bodies" (EB) by the "hanging drop" method (Robertson, 1987; Wobus et al., 2002) or by "mass culture" (Doetschman et al., 1985). The "hanging drop" method generates EBs of defined cell number, and is recommended for developmental studies, because the differentiation pattern is dependent on the number of ES cells that differentiate within the EBs. To produce a high number of EBs, a technology for the controlled generation of EBs in a large scale was established (Dang et al., 2002). For the generation of cardiac, neuronal or pancreatic cells, special culture conditions and protocols are required:

(1) The differentiation of cardiac cells includes three principal steps: (i) the cultivation of cells in "hanging drops" to form EBs; (ii) the cultivation of EBs in suspension; (iii) plating of 5–7 days old EBs and further differentiation/specialisation of cardiac cells (for details see Boheler et al., 2002; Wobus et al., 2002).
(2) For neuronal differentiation, various protocols have been established, including the differentiation induction by retinoic acid (RA), lineage-restricted differentiation and neural induction by stromal cell-derived inducing activity (rev. Gottlieb, 2002). Here, we present a strategy for the lineage-restricted neuronal differentiation that includes the following four steps: (i) formation of cells of all three primary germ layers in EBs; (ii) selective differentiation of neuroectodermal and inhibition of mesodermal cells by growth factor removal (serum depletion); (iii) induction of proliferation of neural precursor cells by bFGF and EGF; (iv) induction of differentiation and maintenance of functional neurons by withdrawal of bFGF/EGF and cultivation in the presence of specific neuronal differentiation and maturation factors (see Rolletschek et al., 2001).
(3) The differentiation of ES cells into pancreatic endocrine cells has been described only recently. Spontaneous differentiation led to the generation of cells expressing genes characteristic for pancreatic islets (Assady et al., 2001; Shiroi et al., 2002; Blyszczuk et al., 2003), but these cells could not be further differentiated into functional beta-like cells. Therefore, we have developed a three-step strategy to generate pancreatic endocrine cells: (i) formation of cells of all three primary germ layers in EBs; (ii) selective differentiation of pancreatic progenitor cells; (iii) the induction of differentiation into functional islet-like clusters by specific pancreatic differentiation factors, such as nicotinamide, laminin and insulin (for details see Blyszczuk et al., 2003).

In addition, constitutive expression of tissue-specific genes ('gain-of-function') in ES cells is another strategy to increase the efficiency of differentiation into cardiac, neuronal and pancreatic cell types (Li et al., 1998; Jackson et al., 2002; Blyszczuk et al., 2003).

2.2. Reverse transcriptase (RT)-PCR analysis

Tissue-specific gene expression patterns are analysed by RT-PCR. The preparation of cell samples,

isolation of total RNA, RT reactions and amplifications are standard procedures and were done as described (Chomczynski and Sacchi, 1987; Rolletschek et al., 2001; Wobus et al., 2002; Blyszczuk et al., 2003).

2.3. Immunofluorescence analysis

For the characterisation of ES cell-differentiated phenotypes, immunofluorescence analyses using antibodies against tissue-specific proteins, such as intermediate filaments, sarcomeric proteins or neurotransmitters are applied according to standard protocols (see Rolletschek et al., 2001; Wobus et al., 2002; Blyszczuk et al., 2003).

2.4. Functional analyses of ES cell-derived cardiac and neuronal cells by electrophysiological methods

Functional properties of differentiated cells may be characterised by electrophysiological techniques using the whole-cell patch-clamp technique. Such methods led to the characterisation of action potentials and ion channels of cardiac (Maltsev et al., 1994, 1999) and neuronal cells (Strübing et al., 1995), whereas the electrophysiological properties have to be established for ES-derived pancreatic cells.

2.5. Toxicity studies with ES cell-differentiated phenotypes

To analyse the cytotoxic and differentiation inhibitory effects of the embryotoxic compound retinoic acid (RA), ES cells were differentiated in the presence of RA at different times and with different concentrations, and developmental effects on the formation of cardiac, skeletal muscle and neuronal cells were investigated (see Wobus et al., 1994b; Rohwedel et al., 2001).

These results showed that in principle, cytotoxic/embryotoxic factors could be analysed in vitro for their potential embryotoxic effects. Based on these studies, the so-called embryotoxicity test (EST, Spielmann et al., 1997) was established. Until now, compounds of different chemical groups have been analysed (for detailed methods and statistical analysis, see Scholz et al., 1999).

2.6. Cytotoxicity tests using dopaminergic neurons

To analyse toxic effects of the cytotoxic compound *N*-methyl-4-phenyl- 1,2, 3,6- tetrahydropyridinehydrochloride (MPTP) in dopaminergic neurons, ES-derived cultures containing dopaminergic neurons and astrocytes were incubated with 100 μM of MPTP for 48 h and immunofluorescence analyses using antibodies against tyrosin hydroxylase (TH) and glial fibrillary acidic protein (GFAP) were carried out (see Rolletschek et al., 2001).

2.7. Functional analysis using ES cell-derived pancreatic cells

Toxic effects of specific drugs or test substances on ES-derived pancreatic endocrine cells could be analysed by measuring intracellular and secreted insulin levels. Glucose-dependent insulin secretion in response to agonists, such as tolbutamide, has been investigated by ELISA using ES-derived pancreatic endocrine cells (see Blyszczuk et al., 2003).

3. Results and discussion

3.1. Cardiac cells

ES cells differentiate efficiently into functional cardiac cells representing the three major cardiac cell types, such as pacemaker-, atrium- and ventricle-like cells. Embryonic pacemaker-like cells represent the earliest cardiac cell type followed by the formation of differentiated cell types, such as atrium- and ventricle-like cells. The first spontaneously beating clusters in EBs can be seen in 7 day old EBs, or about 2 days after plating 5 day EBs (Wobus et al., 1991; Maltsev et al., 1993). The maximum level of beating cardiomyocytes is achieved 7–10 days after EB plating.

During differentiation, a characteristic cardiac-specific gene expression pattern was observed. Nkx 2.5, α- and β-MHC and α_1 CaCh mRNAs were found at the early differentiation stage (Fig. 1A, see Guan et al., 1999), whereas ANF (atrium-like cells) and MLC-2v mRNAs (ventricle-like cells) were detected only at terminal stages.

Also, the structural organisation of sarcomeres in ES-derived cardiomyocytes followed a controlled differentiation program beginning at the Z-band by the organisation of titin (Z-disk epitop; see Fig. 1A), desmin, α-actinin, myomesin, MHC and α-actin. M-proteins at the M-band of sarcomeres are organised only at terminal stages (Guan et al., 1999).

Because the three different cell types express specific ion channels, which produce unique action potentials, they can be defined by patch-clamp analysis (Wobus et al., 1997). In the cardiac specialisation stage, around 50% ventricle-, 40% atrium-, and 10% pacemaker-like cells exist in ES-derived outgrowths.

These ES-derived cardiac cells provide a valuable tool for estimating the pharmacological or cardiotoxic potential of compounds in vitro. The stimulation of the L-type Ca^{2+} current (I_{CaL}) by β-adrenergic agonists is an important process in regulating heart rate and excitation–contraction coupling. Therefore, the modulation of I_{CaL} was used as a functional assay to test different components of the β-adrenergic signalling cascade (Maltsev et al., 1999).

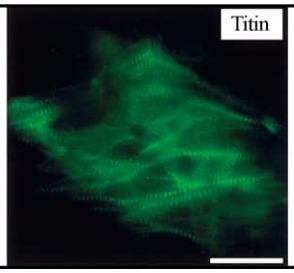
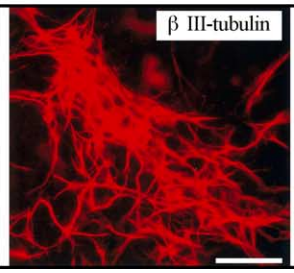
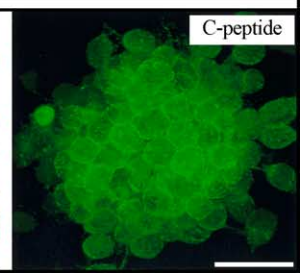
Further, the effects of cytotoxic or embryotoxic compounds have been analysed for their effects on the differentiation of ES cells with the so-called "embryonic stem cell test" (EST) that includes the differentiation of ES cells in the presence of cytotoxic/embryotoxic compounds into cardiomyocytes (Spielmann et al., 1997; Scholz et al., 1999). Parameters, such as the inhibition of ES cell differentiation into cardiomyocytes after 10 days of treatment, and effects of the test substances on the viability of ES cells were determined (Scholz et al., 1999). By application of the "improved prediction model", the potential of chemicals was classified as non-weakly, weakly or strongly embryotoxic.

In addition, genetically engineered ES cells on the basis of tissue-specific promoters that control reporter genes have been adapted for embryotoxicological studies in vitro. First experiments have been done with RA-treatment of differentiating ES cells containing the beta-galactosidase (reporter) gene under the control of the cardiac ventricle-specific myosin-light chain-2v (MLC-2v) promoter (Wobus et al., 1997). With other reporter systems (green fluorescent protein, GFP) under the control of the cardiac actin promoter, the effects of 15 chemicals on cardiac differentiation were determined by parameters, such as morphology and contractile activity, quantitative mRNA analysis and reporter gene activation (Bremer et al., 2001). These in vitro systems were developed as alternative methods comprehensive to in vivo studies in reproductive toxicology and offered reliable results about toxic effects of chemicals in vitro (see Scholz et al., 1999).

3.2. Neuronal cells

ES cell-derived neuronal differentiation is characterised by the development of neural precursor

Fig. 1. Regulated expression of tissue-specific genes, proteins and ion channels during in vitro differentiation of ES cells. (A) Cardiac differentiation: the cardiac transcription factor Nkx 2.5, α- and β-cardiac myosin heavy chain (α- and β-MHC), the α_1 subunit of the L-type Ca^{2+} channel (α_1CaCh), atrial natriuretic factor (ANF) and the ventricular isoform 2 of myosin-light chain (MLC-2v) were expressed in a coordinated manner. Sarcomeric proteins are structurally organised in the sequence: titin (Z-band), desmin, α-actinin, myomesin, α-cardiac MHC, sarcomeric MHC, α-actin and M-protein. Cardiac-specific ion currents i.e. I_{Ca} (L-type Ca^{2+} current), $I_{K,to}$ (transient K^+ current), $I_{K,ATP}$ (ATP-modulated K^+ current), I_K (outwardly rectifying K^+ current), I_{Na} (inward Na^+ current), I_f (hyperpolarisation-activated pacemaker current), and I_{Kl} (inwardly rectifying K^+ current) were found. Titin-labelled cardiomyocytes are shown at stage 5 + 14 days. (B) Neuronal differentiation: nestin, mash-1, engrailed-1 (En-1), 160 kDA neurofilament protein (NFM), the synaptic vesicle protein synaptophysin, and Nurr1 (specifically expressed in dopaminergic neurons) and tyrosine hydroxylase (TH) were expressed in a regulated pattern during ES cell differentiation. The neuron-specific proteins nestin, NFM, synaptophysin, TH, γ-aminobutyric acid (GABA), serotonin, dopamine and dopamine transporter (DAT) were determined by immunofluorescence analysis. Neuron-specific voltage-dependent ion currents, such as I_K (K^+ current), I_{Na} (Na^+ current) and I_{Ca} (Ca^{2+} current), and neuron-specific receptors, such as $(GABA)_A$, glycin (Gly), kainate (Kai) and *N*-methyl-D-aspartate (NMDA) were found by electrophysiological analyses. ßIII-tubulin-expressing neurons are shown at stage 4 + 23 days. (C) Pancreatic differentiation: early pancreatic differentiation is characterised by the expression of pancreatic developmental control genes sonic hedgehog (shh), islet-1 (Isl-1), Pdx-1 and Pax6. High expression levels of genes specifically expressed in beta-cells including insulin, islet amyloid polypeptide (IAPP) and glucose transporter-2 (Glut-2) were found at terminal stages. ES-derived islets were labelled by the pancreas-specific proteins insulin, glucagon, somatostatin and pancreatic polypeptide (PP). Specific ion currents I_{Na}, I_K, $I_{K,ATP}$ and I_{Ca} were measured (unpublished data). A C-peptide-positive cluster derived from ES cells is shown at stage 4 + 28 days; bar = 40 μm (A, B), bar = 20 μm (C).

	(A) Cardiac differentiation		(B) Neuronal differentiation		(C) Pancreatic differentiation	
Stage	Early	Terminal	Early	Terminal	Early	Terminal
Genes	Nkx 2.5		Nestin		shh	
	α–MHC		Mash-1		islet	
	β–MHC		En-1		Pdx-1	
	α_1 CaCh		NFM		Pax6	
	ANF		Synaptophysin		insulin	
	MLC-2V		Nurr1		IAPP	
			TH		Glut-2	
Proteins	Titin		Nestin		Insulin	
	Desmin		NFM		Glucagon	
	α-actinin		Synaptophysin		Somatostatin	
	Myomesin		TH		PP	
	α-cardiac MHC		GABA			
	Sarcomeric MHC		Serotonin			
	Sarcomeric α-actin		Dopamine			
	M-Protein		DAT			
Ion channels/receptors	I_{Ca}		I_K		I_{Na+}	+
	$I_{K,to}$		I_{Na}		I_K	+
	$I_{K,ATP}$		I_{Ca}		$I_{K,ATP}$	+
	I_K		$(GABA)_A$		I_{Ca2+}	+
	I_{Na}		Gly			
	I_f		Kai			
	I_{Kl}		NMDA			
Tissue-specific proteins						

cells that selectively express the intermediate filament protein nestin in about 70% of the population. At early differentiation stage (4 + 10 days), genes coding for nestin and the neural transcription factors mash-1 and engrailed-1 (En-1; Fig. 1B) were expressed (Rolletschek et al., 2001). With ongoing differentiation and under the influence of neuronal differentiation factors, nestin expression is down regulated and neuron-specific transcripts and proteins appeared. At terminal stages, NFM and synaptophysin transcripts as common neuronal markers, and Nurr1 and tyrosine hydroxylase (TH) transcripts as specific markers of dopaminergic neurons were detected. About 40–50% ßIII-tubulin-positive neurons (see Fig. 1B), 20% TH- (see Fig. 2B) and 20% dopamine transporter-positive dopaminergic neurons, 15% GABAergic and 10% serotoninergic neurons within the synaptophysin-labelled cell fraction have been found at terminal stages (Rolletschek et al., 2001).

Neuronal differentiation was also characterised by the onset and further increase in the density of the expression of voltage-gated and receptor-operated ion channels (Fig. 1B, see Strübing et al., 1995) and the synthesis of dopamine measured by HPLC (Rolletschek et al., 2001).

Because ES-derived terminally differentiated neurons resemble functional properties similar to primary cultures (Strübing et al., 1995), but show a higher viability and form complex neuronal/glial networks when cultured under appropriate conditions (see Rolletschek et al., 2001), the cells offer the opportunity to test neurotoxic substances, such as MPTP (Fig. 2A). The effects of MPTP have been investigated in vivo (Przedborski and Jackson-Lewis, 1998) and on primary cell cultures in vitro (Marini et al., 1989). The advantage of the ES cell differentiation system is the formation of GFAP-positive astrocytes (Fig. 2B) in close contact to TH-positive neurons, the requirement for metabolic conversion of MPTP into MPP^+. The significant loss of TH-positive neurons after incubation with MPTP (Fig. 2C) demonstrated that (i) GFAP-positive astrocytes revealed monoamine oxidase B activity and (ii) dopamine transporter is functionally active (see Rolletschek et al., 2001).

ES-derived dopaminergic neurons characterised by high survival capacity in vitro could represent a reliable screening system to test neurotoxic compounds, or drugs for the treatment of patients suffering from Parkinson‘s disease, a neurodegenerative disorder caused by a dramatic loss of dopaminergic neurons (Fahn, 1989).

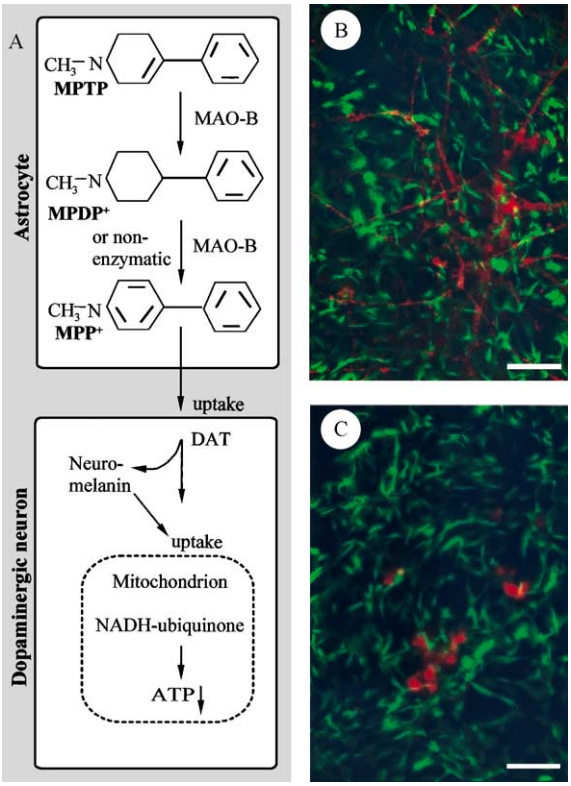


Fig. 2. MPTP-induced neurotoxicity on ES cell-derived dopaminergic neurons. (A) To express its cytotoxic effects, MPTP has to be converted to the toxic metabolite MPP^+ by astrocytic monoamine oxidase B activity. Specific catecholamine uptake sites on terminals and cell bodies of dopaminergic neurons accumulate MPP^+, where it decreases the production of ATP and increases the formation of free radicals. Subsequently, energy failure and oxidative stress lead to cell damage of dopaminergic neurons. (B) Control cultures without addition of MPTP displayed a high number of TH (red)-positive dopaminergic neurons and GFAP (green)-positive astrocytes. (C) Application of MPTP for 2 days at late differentiation stage caused cell shrinkage and damage of neuronal networks of TH (red)-positive neurons but not of GFAP (green)-positive astrocytes (according to Rolletschek et al., 2001); bar = 25 μm.

3.3. Pancreatic cells

Pancreatic differentiation of ES cells is characterised by the expression of pancreatic developmental

control genes at early differentiation stages followed by the up-regulation of genes specifically expressed in beta-cells at terminal stages (Fig. 1C, Blyszczuk et al., 2003).

Cells co-expressing C-peptide and insulin are organised in islet-like clusters (Fig. 1C), and electrophysiological studies showed activity of beta-cell specific ion channels (unpublished data). The cells formed insulin-positive secretary granules, released insulin in response to high glucose concentrations and normalised blood glucose levels in diabetic mice (Blyszczuk et al., 2003).

Permanent cell lines or primary cultures of beta-cells have been used to analyse cytotoxic substances, such as streptozotocin, commonly used to induce diabetes in animal models and alloxan, which acts via the induction of free radicals resulting in the destruction of beta-cells (Szkudelski, 2001).

Another approach may be the investigation of insulin release by secretagogues, such as sulphonylureas used for the treatment of type 2 (non-insulin dependent) diabetes by stimulation of insulin secretion. The mechanism of glucose-dependent insulin secretion and signalling pathways are well characterised and include the activity of ATP-dependent K^+ channels (rev. Henquin, 2000). Most of the today available drugs, such as tolbutamide (stimulating insulin release) and diazoxide (inhibiting insulin release), affect the ATP-dependent K^+ channel activity in pancreatic beta-cells (Trube et al., 1986), insulin-secreting cell lines (Dunne et al., 1987; rev. McClenaghan and Flatt, 1999), but also in ES-derived pancreatic cells (Lumelsky et al., 2001; Blyszczuk et al., 2003).

Established pancreatic cell lines used to analyse the molecular mechanism of insulin release do not represent functional islets, and thus toxic effects of drugs on beta-cell development cannot be analysed. Because ES cells are able to generate functional islet-like clusters in vitro (Blyszczuk et al., 2003), the cells may be suitable for such investigations, if the insulin secretion machinery in ES-derived pancreatic cells has been established.

4. Conclusions

At present, applications using ES cells in basic and applied toxicology are still preliminary. The examples presented here, demonstrate the use of the ES cell technology to analyse pharmacological and toxicological effects of drugs and xenobiotics in vitro. Future developments and technical inventions will further improve the application of the ES cell technology in basic and applied toxicology.

These technical and methodological developments include: (i) genomics and proteomics techniques for the identification of target genes and signalling molecules that are modified after application of test compounds to ES cells and their differentiated derivatives (Anisimov et al., 2002); (ii) new strategies for the genetic and epigenetic manipulation of cells, such as the iRNA technology (Elbashir et al., 2001); (iii) for protein transduction systems (Elliott and O'Hare, 1997; Schwarze et al., 1999). Finally, the application of these new technologies to human ES cells in the future will substantially affect toxicology research.

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