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: Hlx9, Hnf6, Hnf3beta and Pdx1. Hlx9 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<sub>ATP</sub>) channels. It results in membrane depolarisation (ΔΦ) and opening of voltage-dependent calcium channels (VDCC). The subsequent increase in cytosolic free Ca<sup>2+</sup> 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. Acetylcholine (ACh) and cholecystokinin (Cck) promote phosphoinositide breakdown with a consequent mobilisation of Ca<sup>2+</sup> 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<sub>ATP</sub> channels, thus inducing insulin secretion even at low glucose concentration, whereas nifedipine is a blocker of Ca<sup>2+</sup> 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