GENE THERAPY FOR DIABETES

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1. ABSTRACT

For more than eighty years, insulin injection has been the only treatment option for all type I and many type II diabetic individuals. Whole pancreas transplantation has been a successful approach for some patients, but is a difficult and complex operation. Recently, it was demonstrated that a glucocorticoid-free immunosuppressive regimen led to remarkably successful islet transplantation. However, both pancreas and islet cell transplantation are limited by the tremendous shortage of cadaveric pancreases that are available for transplantation. Therefore, a major goal of diabetes research is to generate an unlimited source of cells exhibiting glucose-responsive insulin secretion that can be used for transplantation, ideally without the need for systemic immunosuppression. The focus of this review is on how gene therapy can be used in beta cell replacement strategies. Gene transfer to beta cells as well as recent advances in beta cell growth and development will be discussed.

2. INTRODUCTION

The goal of diabetes mellitus therapy is to maintain normoglycemia in the face of variations in dietary intake. The Diabetes Control and Complication Trial (DCCT) has shown that tight glucose control is necessary in order to lower the incidence of diabetic complications. However, tight glucose control by multiple insulin injection leads to an increase in the number and severity of hypoglycemic episodes due to the absence of an ideal glucose sensing system coupled to insulin administration (1). Although much effort has been devoted to the development of an artificial glucose sensor, there are still substantial technical obstacles that need to be overcome (2-4). Thus, cell transplantation therapy may be the best solution for the restoration of normal physiological glucose control.

Pancreatic islet transplantation has been a subject of study for the last thirty years (5). However, it was not until the 1990's that the first successful procedures were reported (6). In 1996, the Islet Transplant Registry estimated that only 6 percent of patients who received islet transplantation in the years from 1990 to 1995 were free from insulin treatment for up to a year (7). The reason for this was thought to be due at least in part to toxic effects of the immunosuppressive drugs, particularly steroids, on beta cell function. Recently, it was shown that a glucocorticoid-free immunosuppressive regimen allowed successful islet transplantation in patients with type 1 diabetes who had a history of severe hypoglycemia and metabolic instability (8). Seven of seven patients maintained normal blood glucose concentrations and glycosylated hemoglobin values without exogenous insulin for an average of one year. This study represents a breakthrough since it demonstrated for
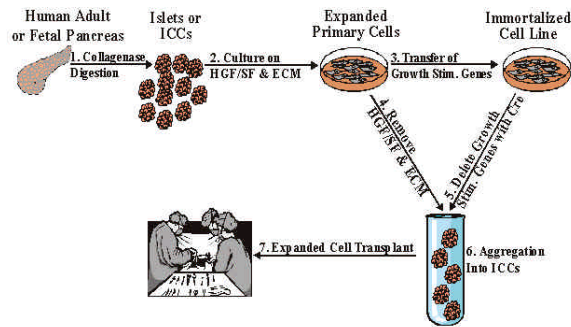
Figure 1. Strategies for expanding human beta cells. 1) Limited collagenase digestion releases islets from a cadaveric human pancreas. 2) beta cells are induced to divide using hepatocyte growth factor (HGF/SF) and a complex extracellular matrix (ECM). 3) Retroviral vectors are used to transfer growth stimulatory genes into the dividing beta cells to create immortalized cell lines. 4&6) Expanded primary cells can be induced to withdraw from the cell cycle and differentiate by growing the cells as aggregates in suspension and removing HGF/SF. 5&6) Immortalized cell lines can be induced to withdraw from the cell cycle and differentiate by deleting the growth stimulatory gene using the cre-lox recombinase system and growing the cells as aggregates. 7) Aggregated cells are transplanted *in vivo*.

the first time that islet transplantation could consistently cure diabetes. Larger scale clinical trials using a similar protocol are in progress at an expanded number of islet transplant centers around the world. However, to achieve a cure for the millions of patients with insulin-dependent diabetes, the lack of a sufficient number of cadaveric donors, as well as the autoimmune and allograft rejection after transplantation, have to be overcome. Thus, there is a need to generate an unlimited source of cells with glucose responsive insulin secretion, ideally without requiring systemic immunosuppression.

3. CELL TRANSPLANTATION THERAPY FOR DIABETES

There is an imbalance between the supply and demand for cadaveric pancreases to be used as a source of tissue for transplantation in individuals with diabetes. In the USA, there are ~30,000 new cases of type 1 diabetes each year (9), as well as a considerable number of patients with insulin dependent type 2 diabetes who might be candidates for beta cell replacement. However, only ~5,000 brain-dead organ donors are available each year, and just some of these can be used as a source of pancreatic tissue (10). To solve this problem, other sources of cells are being studied as candidates for cell based diabetes therapy.

Beta cells from human or non-human sources are the obvious source of cells for beta cell replacement strategies. The use of xenogeneic beta cells, particularly from pigs, is being studied intensively. Although the supply of tissue would not be an issue, the potent xenogeneic immune response and the potential for endogenous porcine retrovirus infections would have to be overcome (11-15).

Human beta cells are an attractive source of tissue, but require the ability to expand the limited amount of tissue *in vitro* in order to treat the large number of insulin-dependent diabetic patients "figure 1". The expansion of primary beta cells or beta cell lines *in vitro* has been the focus of many studies (16-18). This is a challenge for a number of reasons. First, the signals that trigger beta cell proliferation are incompletely understood. Second, when beta cells are induced to proliferate, they tend to lose differentiated function. Finally, as will be discussed further below, primary beta cells have a very limited *in vitro* lifespan.

Introducing the complex cellular machinery involved in glucose-responsive insulin secretion in non-beta cells represents another option for cell transplantation. This approach has the advantage of providing flexibility regarding the choice of starting cell but has the disadvantage of needing to precisely understand and mimic the incompletely understood machinery involved in glucose-responsive insulin secretion. Therefore, the best source of cells for a successful and widely applicable beta cell replacement strategy is likely to come from human beta cells or beta cell precursors.

4. PANCREATIC BETA CELLS FOR CELL TRANPLANTATION

4.1. Xenogeneic beta cells

Xenotransplantation, the transplantation of organs, tissues or cells between animal species, would provide an unlimited number of beta cells. The pig has been studied as the most suitable animal donor (19-22) and the recent report of pig cloning may have a major impact on the eventual success of xenotransplantation (23). However, it is very difficult to obtain healthy adult pig islets due to their poor survival in tissue culture (24,25). A major problem with the use of pig organs for transplantation is the phenomenon of hyperacute rejection, mediated by preexisting antibodies to an alpha-galactosyl xenoepitope in pigs. Although xenogeneic islets, unlike vascularized solid organs, do not undergo hyperacute rejection, they eventually undergo a delayed xenograft rejection. Delayed rejection does not appear to be alpha-galactosyl xenoepitope dependent (11-13), but is mediated by other undefined xenoantigens (14). Another serious concern regarding xenotransplantation comes from the risk of an infection by endogenous porcine retroviruses that have been shown to infect human cells *in vitro* and *in vivo* (15,26). The breeding of pigs lacking the endogenous retroviruses will be extremely difficult, since multiple copies of the virus are integrated in the animal genome (27).

4.2. Expansion of primary pancreatic beta cells and beta cell precursors

Although beta cells are known to have a limited capacity for replication, hepatocyte growth factor/scattered factor (HGF/SF) in combination with complex extracellular matrices stimulate adult beta cells and beta cell precursors to proliferate (28,29). However, growth stimulation leads to loss of differentiation, with a rapid decrease in insulin expression (29,30). Moreover, primary beta cell expansion

is limited to 10-20 population doublings, after which they undergo growth arrest due to cellular senescence (29,31). It has been demonstrated that expanded primary cells have shortened telomeres, elevated levels of senescence-associated beta-galactosidase, and increased expression of the cyclin dependent kinase inhibitor p16 INK4a (32).

Beta cell precursors have been suggested as a source of tissue for transplantation because they may have an increased proliferative potential (33). However, this has not yet been formally demonstrated. Furthermore, despite great effort directed towards understanding the factors controlling the growth and development of human beta cell precursors, the identity of the physiologically relevant factors remains unknown for the most part (28,34-36). *In vivo*, the neogenesis of endocrine islets from ductal epithelium has been described after various experimental conditions such as 90% pancreatectomy, pancreas wrapping in the rodent, (37,38) or when transplanted together with fetal mesenchyme into nude mice (39). *In vitro,* the neogenesis of endocrine islets from ducts with the use of matrix and growth factors has been suggested as an approach to human islet propagation in order to increase the mass of endocrine tissue obtained from adult cadaveric pancreases for transplantation (40). Recently, it was shown that human ductal cells exposed to Matrigel and growth factors could be directed to differentiate into islet endocrine cells *in vitro*. Under these conditions, the formation of structures that were called cultivated human islet buds (CHIBs) was observed. While this is a promising beginning, it is limited so far by the very limited yield of differentiated cells (41).

Another possible source of cells is embryonal stem (ES) cells, as they have the ability to differentiate *in vitro* into different cell lineages (42-43). It has been shown that an insulin-secreting cell clone from undifferentiated ES cells normalized the glycemia in streptozotocin-induced diabetic mice. The cell clone was obtained by expressing and selecting for a construct with a neomycin dominant selectable marker gene under the control of the human insulin promoter. Surviving cells expressed the endogenous insulin gene and were implanted into the spleen of mice (44). However, 40% of the animals that achieved normoglycemia after implantation developed hyperglycemia 12 weeks after the transplant. Unfortunately in the other 60% of the animals that remained normoglycemic, the spleen was never removed to rule out the possibility of pancreas regeneration. Moreover, it is not clear how the immune response was controlled since no immunosuppression or encapsulation was used.

Ramiya *et al.* have reported the formation of islet cells generated *in vitro* from pancreatic ductal epithelial cells isolated from adult non-obese diabetic (NOD) mice. This study showed that pluripotent stem cells isolated from the pancreatic ducts and maintained in long-term culture were able to reverse insulin-dependent diabetes after transplantation under the kidney capsule of diabetic NOD mice (45). Because the kidney with the graft was not removed, it is impossible to rule out the possibility that the reversal of diabetes was due to the graft and not to

regeneration from autologous cells. It is interesting that, although the transplanted cells came from pre-diabetic NOD mice, the transplanted animals did not develop diabetes. This is a very puzzling finding that, if reproducible, raises the possibility that cultured islets cells from an early diabetic patient could be transplanted back into that patient without rejection.

5. PANCREATIC DEVELOPMENT

As discussed above, the limited replication capacity of mature beta cells makes beta cell progenitors attractive candidates as a source of tissue for transplantation. Using beta cell progenitors, particularly in approaches where differentiation is induced *in vitro*, requires that the process of beta cell growth and development be well understood. The pancreas is generated from the upper duodenal part of the embryonic gut via a dorsal and ventral protrusion of the epithelium directly posterior to the developing stomach. Both formation of the pancreas and the small intestine from the embryonic gut are dependent on intercellular signaling between the endodermal and mesodermal cells of the gut (46-49). Similar to the pancreas in general, pancreatic endocrine cell development involves a complex interaction among extracellular soluble factors, cell-matrix and cell-cell interactions, which will ultimately act through transcription factors. While there has been an explosion of new information about this process, particularly in the identification of transcription factors that promote and maintain beta cell function, large gaps in our knowledge remain. Filling in those gaps is a major priority of diabetes research, as the genes encoding molecules important for beta cell development are potential targets for gene therapy to promote beta cell development from precursors both *in vivo* and *in vitro*.

5.1. Secreted growth and differentiation factors

To develop new sources of insulin-producing cells for transplantation, much attention has been devoted to the extracellular signals that mediate endocrine cell development. Studies in the chick have shown that notochord can repress *sonic hedgehog* (*shh*) expression, allowing for pancreatic differentiation through intercellular signaling molecules, including activin beta B and fibroblast growth factor 2 (50). Other lines of evidence also point to a role for activins in pancreas development. Transgenic mice expressing activin receptor mutants show hypoplasia of pancreatic islets (51). Moreover, follistatin, the activin binding protein, can mimic the repressive effects of the mesenchyme on the differentiation of rat pancreatic endocrine cells (52).

In some cases, activins have been shown to work in concert with other factors to promote endocrine differentiation. Betacellulin was originally isolated from a mouse pancreatic beta cell tumor line and has been shown to promote the proliferation of the rat insulinoma cell line INS-1 (53). It is a member of the epidermal growth factor (EGF) family and is expressed in the human pancreas (54). It was shown that betacellulin and EGF receptor (EGFR) expression are present in the human pancreas and that there

is a disturbed formation of pancreatic islets in mice lacking EGFR (54,55). Moreover, betacellulin has been shown to be required for insulin gene expression in clonal alpha cells transfected with the PDX-1 gene (56). Activin A and betacellulin act synergistically to convert exocrine AR42J cells to insulin expressing cells (57). This was also observed when the exocrine cells were treated with activin A and hepatocyte growth factor (58). However, in human undifferentiated pancreatic epithelial cells, activin A and betacellulin were shown to have distinct effects. Activin A induced endocrine differentiation with an increase in insulin expression while betacellulin promoted proliferation (59).

Hepatocyte growth factor/scatter factor (HGF/SF) is a mesenchyme-derived protein that acts on epithelial cells through a membrane-spanning tyrosine kinase receptor, c-met. HGF/SF and c-met gene expression are highly expressed during early pancreas development, and maintained at low levels during puberty and adult life (60-62). Immunofluorescence studies have demonstrated co-localization of the c-Met receptor protein with insulin-containing cells in the islet. Furthermore, it has been shown that HGF/SF is mitogenic to epithelial cells from the human fetal pancreas (61). HGF/SF alone is also able to convert pancreatic acinar AR42J cells into insulin-producing cells (58). Moreover, HGF/SF overexpression in the islet of transgenic mice leads to an increase in beta cell proliferation, islet mass and to mild hypoglycemia (63). In addition, HGF/SF was shown to increase the expression of Reg, a protein implicated in pancreatic regeneration, in human fetal islets (64).

Other factors that may play important roles in endocrine growth and differentiation include prolactin, which is a potent activator of islet cell growth *in vitro* (65) and glucagon-like peptide 1 (GLP-1) and its more stable analog exendin-4. GLP-1 induced AR42J cells to differentiate into insulin, pancreatic polypeptide and glucagon positive cells (66), and exendin-4 stimulated beta cell replication and neogenesis in diabetic rats (67). Moreover, activation of the GLP-1 receptor was shown to act synergistically with the transcription factor PDX-1 and cell-cell contact to activate insulin gene in a human beta cell line (68).

5.2. Extracellular matrix and cell adhesion factors

It is known that tissue differentiation during development depends on the expression of molecules that regulate cell-cell and cell-matrix interactions (69,70). Cell adhesion molecules from the CAM immunoglobin superfamily and the calcium dependent cadherin family have been shown to be important in the development of pancreas (71,72). Moreover, connexin 43 gene expression promoted insulin gene expression in rat insulinoma cells (73). Extracellular matrix has been found to play a role in *ex-vivo* expansion of human pancreatic endocrine cells (28), although the signaling pathway involved has not been identified. Cell-cell contact can induces differentiation in pancreatic primary cells (30) and also in human pancreatic cell lines when acting in synergy with the homeodomain transcription factor PDX-1 (74).

5.3. Transcription Factors

Inducing beta cell differentiation from precursors or extrapancreatic cells by the expression of transcription factors that are necessary for beta cell development is one of the possible approaches to gene therapy for diabetes. There has been an explosion in our understanding of the transcription factors that are involved in beta cell development, but large gaps remain (75-80).

The homeodomain transcription factor PDX-1 (also known as IDX-1, IUF-1, STF-1, and IPF-1) is expressed in the epithelium where pancreatic evaginations appear (81). Homozygosity for mutations in the PDX-1 gene in mice and humans results in pancreas agenesis (76,82). Although PDX-1 is not required for bud formation, its absence compromises pancreatic epithelial proliferation, branching and differentiation (75-80,83,84). In mature beta cells, PDX-1 transactivates the insulin gene and other fundamental islet cell genes as GLUT2, glucokinase, islet-amyloid polypeptide (IAPP) and somatostatin (85,86). Heterozygosity for mutations in the human PDX-1 gene leads to maturity-onset diabetes of the young (MODY 4) (87). Thus, PDX- plays a dual role, being essential early in development for the development of the entire organ as well as late in development for beta cell function. To test the idea that PDX-1 might be a master regulator of beta cell differentiation, an adenoviral vector was used to express PDX-1 in rodent liver in vivo. While this resulted in the generation of a small number of insulin-positive cells, the origin of those cells and whether they exhibit glucose-responsive insulin secretion is not yet known (88).

Transcription factors other than PDX-1 that are important in beta cell differentiation and function cause other forms of MODY. MODY 1 and 3 are caused by mutations in hepatocyte nuclear factor (HNF)-4 alpha and –1alpha, respectively; MODY 5 to mutations in HNF-1 beta. MODY 2 is an outlier, being caused by mutations in the glucose-sensing enzyme glucokinase (89-91). ISL1, a LIM-homeodomain transcription factor, is important in pancreas development. Mice lacking ISL1 do not develop the dorsal pancreatic mesenchyme and have a failure of exocrine cell differentiation in the dorsal, but not the ventral, pancreas. There is also a complete loss of differentiated islet cells (75). Genes of the Pax family are important for endocrine pancreatic cell differentiation. Mice that are homozygous for Pax 6 gene deletions have a reduced number of all differentiated cell types and hormone production (79,83,92). In contrast, Pax 4 gene knockout mice fail to develop beta and delta cells associated with alpha cell hyperplasia (78). Some of the NK-homeodomain transcriptional factors have been shown to have a crucial role in beta cell development. Nkx 2.2 (-/-) mice do not have insulin cells and have a reduced number of glucagon and pancreatic polypeptide secreting cells. Nkx2.2 is required for the development of both the early insulin-expressing cells and the later mature beta cells in contrast to Pax4 and Nkx6.1 (80). Nkx 6.1 is expressed specifically in beta cells and is activated when islet tumor cells assume an insulin-producing phenotype (77,93). Studies in rats demonstrated that Nkx6.1 is important in pancreatic development and in mature insulin cell function (94).

Recently, using transgenic mice doubly mutated in Nkx6.1 and Nkx2.2, it was demonstrated that Nkx6.1 acts downstream of Nkx2.2 in pancreatic beta cell development (95).

NeuroD/Beta2 is a basic helix-loop-helix (bHLH) transcription factor expressed in pancreatic endocrine cells as well as in the central nervous system (96,97). In NeuroD/Beta2-deficient mice, the endocrine cells appear with reduced number, especially beta cells (98). The expression in the endocrine pancreas of genes such as NeuroD/Beta2 that are also present in the nervous system played a role in the mistaken suggestions that there may be a common embryological origin for those cell types. Notch signaling, which plays a critical role in neural development, is important in the development of pancreatic endocrine cells. Mice lacking the Notch ligand Delta-like gene (1Dll1) (99) or the intracellular mediator Rpb-jk (100) have an increased number of endocrine cells in the pancreas (101). A similar phenotype was found in mice over-expressing neurogenin 3 (ngn 3) or the intracellular form of Notch3, a repressor of Notch signaling (102-103). On the other hand, loss of neurogenin 3 function in mice results in a complete absence of endocrine cell differentiation (104). It has been demonstrated that the first step of pancreatic epithelial cells towards an endocrine phenotype is controlled by neurogenin 3. The bHLH proteins are antagonized by the Notch pathway, partly due to a Hairy and Enhancer-of-split/HES-type protein, Hes-1, which encodes a bHLH transcriptional repressor (105). Mice deficient in Hes1 have severe pancreatic hypoplasia due to depletion of pancreatic epithelial precursors caused by accelerated differentiation of post-mitotic glucagon-expressing cells. Thus, Hes-1 acts as a negative regulator of endodermal endocrine differentiation (106).

6. PANCREATIC ENDOCRINE CELL LINES

The generation of functional beta cell lines could overcome the shortage of tissue for transplantation without the need for complex matrices and growth factors to stimulate *in vitro* expansion. Cell lines are a more reproducible source when compared with islets and can be modified by gene transfer in culture to further improve their properties. There has been great interest in beta cell lines from a basic science point of view, as they represent an excellent tool to elucidate the mechanisms involved in glucose responsiveness, insulin gene transcription and for isolating pancreas-specific genes (107-109).

Significant progress has been made in developing rodent pancreatic cell lines (107,108,110-112). Therefore, most work with beta cell lines has been focused on mice, rats and hamsters. The sources for those cell lines include: spontaneous insulinomas (113), carcinogen-induced insulinomas (114), oncogenic virus-induced insulinomas (115-116), insulinomas induced by a combination of carcinogen treatment and oncogenic viruses (115), and insulinomas from transgenic mice expressing dominant oncogenes, particularly SV40 T antigen under the control of the insulin promoter (117-119). The most studied pancreatic cell lines are those derived from a radiation-induced, transplantable insulinoma found in inbred New England Deaconess Hospital (NEDH) albino rats (114),

including the RIN (120), MSL (121), INS (110), and CRI (122) series of cell lines.

While rodent cell lines have served as excellent model systems in many ways, they do have some limitations with respect to their usefulness in both basic and clinical application. Rodent beta cells have some relevant biologic differences when compared to human beta cells. For example, there are reports that rodent and human beta cells differ in their response to inflammatory cytokines (123). There may be differences in the response to growth factors such as HGF/SF (28,35,124,125). Rodents have two copies of the insulin gene instead of the single human insulin gene, and there are differences in pattern of gene regulation compared to the human insulin promoter (126). The GLUT 2 gene, which is a critical glucose transporter on the surface of rodent beta cells, is expressed at much lower levels in the human beta cell and may not be an important glucose transporter in those cells (127,128). These biologic differences and the problems with xenogeneic transplantation have led to an increased interest in developing human beta cell lines.

The development of human pancreatic endocrine cell lines has been a difficult task (129-132). Unlike the case with rodents, it has proven to be extraordinarily difficult to develop cell lines from spontaneous human insulinomas (129,133-138). Human pancreatic endocrine tumors are rare and it has been shown to be difficult to adapt primary human pancreatic endocrine tumors to culture *in vitro* (139). Moreover, there is only a single report of a cell line derived from a human insulinoma (132). Thus, most of human beta cell lines development has been made with the use of oncogenes to induce replication of beta cells (129,130,140,141).

There are some advantages to using oncogenes as an approach for cell line development. First, it is possible to have some control over the starting primary cell from which the cell line will be derived. Second, the oncogenes used to develop the cell line are known, and thus able to have defined and somewhat controllable effects on the growth and differentiation of the resulting cell line. The dominant oncogenes SV40 T antigen and H-ras$^{val\ 12}$ have been used to develop cell lines from human beta cells and beta cell precursors (130,141,142). Although the introduction of these oncogenes enables the cells to replicate beyond their expected lifespan (143), they still face a proliferative block known as "crisis". This can lead them to stop growing or to die. In order to overcome crisis, which is a delayed form of cellular senescence (31), the strategy of induction of telomerase activity in the cell lines by gene transfer of the reverse transcriptase component of the human telomerase (hTRT) together with the oncogenes was used and has been shown to provide immortalization (31).

While the use of oncogenes to develop cell lines leads to indefinite growth, it has profound effects on the ability of the cells to differentiate. However, we have shown that a human cell line, TRM-6, derived from human fetal islets, is able to differentiate along the delta cell lineage upon expression of the transcription factor PDX-1
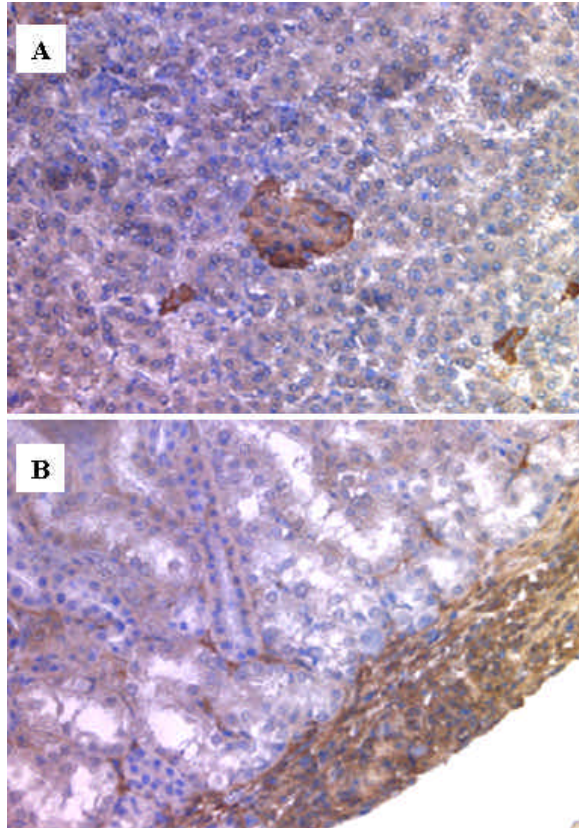
Figure 2. Light micrograph of human adult pancreas immunostained for insulin (brown) (A), and of beta lox5PDX aggregates exposed to exendin 4 *in vitro* for 4 days and transplanted under the kidney capsule of nude mice. The graft was harvested after 4 weeks and immunostained for insulin (brown) (B).

and promotion of cell-cell contact (74). Recently, we showed that betalox5, a cell line derived from purified adult beta cells can be induced to exhibit glucose-responsive insulin secretion *in vitro* and *in vivo* (68) "figure 2". This is the first report of a functional human beta cell line and provides proof of the principle that a human beta cell line can be used for a cell-based therapy for diabetes.

One of the potential drawbacks of grafting transformed and/or immortalized cell lines *in vivo* is the risk of tumor formation. Both rodent (107,144) and human (129,130,140,141) transformed beta cells have shown some phenotypic instability and de-differentiation. This is due to the fact that transformed cells may have their differentiated functions turned off while turning on those functions necessary to cell proliferation. Additionally, the phenotypic changes may come together with the inherent genetic instability of transformed cells. In order to solve this problem, some approaches have been developed based in the idea of turning oncogene expression off after sufficient cell numbers have been obtained. To this end, conditional promoters have been used to regulate the expression of the SVT40 antigen oncoprotein in beta cells (145). The promoter used was based on the bacterial tetracycline (tet)

operon (146). Other inducible promoters (140,141,145,147) as well as the cre-lox recombinase system, which mediates complete deletion of the introduced oncogenes (31,148,149), have also been used to regulate oncogene expression. Immortalized human hepatocytes had the SV40T gene completely excised by cre-lox site-specific recombination before transplantation into the spleen of rats (150). The incorporation of "suicide" genes such as the herpesvirus thymidine kinase (TK) gene to make cells susceptible to killing with ganciclovir (151) has been used to selectively eliminate cells that escape from growth control (152,153).

7. GENE TRANSFER TO PRIMARY BETA CELLS

Gene delivery into primary beta cells has to meet a number of requirements, including the expression of transgenes within post-mitotic cells, the potential need to target a specific cell type within the islet, and the need of achieving an optimal duration of transgene expression. Non-viral methods of gene transfer, such as calcium-phosphate mediated transfection, are inefficient and limited to delivery into actively proliferating cells *in vitro*. Several kinds of viruses have been manipulated for gene transfer and gene therapy applications and there are some advantages and disadvantages regarding each system (for review see (154). Adenoviral vectors do not integrate into the host cell genome and can induce potent immune response against structural proteins, leading to transient expression (155), although they can infect non-dividing cells efficiently. Conventional retroviral vectors cannot infect non-dividing cells (156) but can stably integrate into the host cell genome of dividing cells. More recently, lentiviral vectors such as those based on the human immunodeficiency virus (HIV), have been shown to infect and express genes in both mitotic and post-mitotic cells (157,158).

7.1. Transduction of pancreatic islets with vectors based on DNA viruses
Adenovirus vectors have been used to transfer genes into a variety of endocrine cells like pituitary cells (159), thyroid cells (160) and beta cells. It has been shown that they can transfer genes into pancreatic islets from different species including pigs (161), rodents (162-164) and humans (165). An adenoviral vector carrying the leptin gene have been used to correct the phenotype of the ob/ob mouse, with normalization of food intake, body weight, serum insulin concentration and glucose tolerance (166). Experiments in which Zucker rat pancreas was exposed to adenovirus vectors containing the leptin receptor have shown restoration of islet function (167). Intravenous injection of adenoviral vectors carrying a modified proinsulin cDNA resulted in insulin expression and a reduction in the blood glucose in diabetic mice, but the insulin secretion was constitutive, not glucose-responsive (168).

In vivo and *ex vivo* transduction of pancreatic cells from rats or mice has been described after exposure to recombinant adenovirus vectors encoding hexokinase I or betagalactosidase (162,163). Direct *in vivo* approaches have been tested in rats, using the pancreatico-biliary duct with
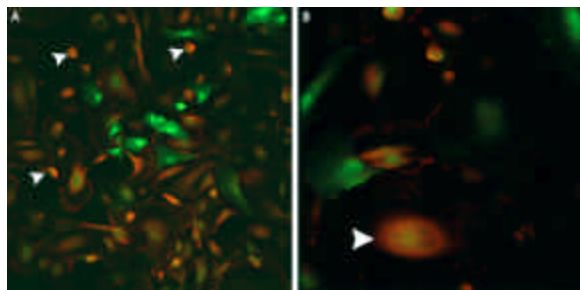
Figure 3. Confocal microscopic analysis of cultured adult human pancreatic cells infected with a lentiviral vector expressing GFP (green) and immunostained for insulin (red). Arrowheads indicate cells with colocalization of GFP and insulin at low (A) and high (B) power.

transduction into the ductal epithelium, acinar cells and islets (169). However, that was associated with pancreatitis and determined only transient gene expression. Although infection with adenoviral vectors does not have a direct effect on insulin secretion, the vectors induce a potent inflammatory response in both ex vivo (infection of islets prior to grafting) (170) and after direct in vivo adenovirus vector-mediated gene transfer (164,171). Antibody production against the transgene carried by the vector is also a problem in some cases (164). Immune response to viral antigens and transgenes is responsible to a substantial degree for the reduction of transgene expression that is commonly observed several weeks after infection with adenoviral vectors (172). Nonimmunologic mechanisms may also be involved since transient gene expression was observed in pancreatic islets transplanted to nude mice (164). The immune response generated against first generation (E1 deleted) adenoviral vectors has led to attempts to delete more genes in the adenovirus genome in order to reduce endogenous gene expression. New generations of so-called "gutless" adenoviral vectors from which all viral genes have been deleted represent a promising approach for in vivo gene expression, but have not yet been applied to the pancreas (173-175).

Adeno-associated viruses (AAV), members of the parvoviridae family, have been used to transfer the leptin gene to correct the endocrine dysfunction in ob/ob mice. This study achieved long-term correction of body weight, food intake, insulin and glucose concentrations, with a normal glucose tolerance test (176). A recombinant AAV vector has been used to infect the liver of diabetic mice with the rat insulin gene, resulting in a decrease in serum glucose concentrations (177). However, similar to the adenovirus studies, insulin secretion was not glucose-responsive. An improvement on that approach used an engineered single chain insulin driven from the glucose-responsive pyruvate kinase promoter. Infection of liver cells with a large amount of virus resulted in glucose-responsive insulin secretion sufficient to substantially cure diabetes in NOD mice, albeit with delayed kinetics compared with secretion from beta cells (178).

7.2. Transduction of pancreatic islets with vectors based on RNA viruses

Retroviral vectors derived from murine retroviruses, particularly Moloney murine leukemia virus, have been the most widely used gene transfer vectors,

particularly in human clinical trials (183). Recombinant retroviral vectors lack all retroviral genes, which are replaced with the marker and/or therapeutic genes. The drawbacks of murine retroviral vectors include: the inability to transduce non-dividing cells, random insertion into the host genome, low titers, and the potential shut-off of transgene expression over time. However, they can mediate long-term transgene expression in humans, stably transduce dividing cells, and do not express viral genes that can trigger immune responses (184). Although Mo-MLV retroviral vectors are not the best choice for *in vivo* gene transfer to the endocrine system due to the low rate of proliferation of most endocrine cells, they are useful for transduction of a wide variety of cell types *in vitro* including anterior pituitary AcT20 cells (185), thyroid cells (186,187), and pancreatic cells (165).

To overcome some of the problems with the murine vectors, particularly the inability to infect non-dividing cells, vectors derived from lentiviruses are becoming increasingly popular. The human immunodeficiency virus (HIV) is the best known lentivirus. Unlike murine retroviral vectors, lentiviral vectors can infect non-dividing cells such as beta cells and integrate into the cell genome. They share with murine retroviral vectors the advantage of being relatively non-immunogenic compared with adenoviral vectors (157,158,188). In a study comparing different viral vectors for their ability to infect human adult and fetal pancreatic endocrine cells infection, lentiviral vectors infected a higher percentage of pancreatic beta cells when compared with murine retroviral vectors "figure 3" (165,189). Recently, a lentiviral vector was used to deliver and express significant levels of soluble interleukin-1 receptor antagonist protein in intact islets. There was no impairment of glucose stimulated insulin secretion following lentiviral infection of islets (190).

7.3. Nonviral Gene Transfer to Pancreatic Islets

Gene delivery using nonviral systems is generally thought of as safer than using vectors derived from pathogenic viruses (172). However, non-viral methods are almost invariably less efficient than viral vectors (191). A variety of non-viral methods of gene transfer have been applied to islet cells, including calcium phosphate coprecipitation (129), and mono- and polycationic lipids such as lipofectin and lipofectamine (192,193). Lipofection is a simple and relatively non-toxic technique that can lead to the expression of a transgene in approximately half of cells from rodent and human islets when the islets were dispersed in a single cell suspension (192). Less common nonviral delivery approaches like electroporation and biolistic transfection have been tested in pancreatic islets (for review see 16). However, there is no apparent advantage when comparing these methods with lipofection, which is a simpler and more efficient technique.

8. ENGINEERING OF GLUCOSE RESPONSE IN NON-BETA CELLS

The scarcity of donors for transplantation and the risk of a recurrent autoimmune response to transplanted islets are the two main reasons why non-beta cells have

been studied as a source of tissue for cell-based therapy for diabetes (194-197). The major obstacle to this approach is the need to reproduce beta cell function in non-beta cells, particularly the process of proinsulin synthesis, processing and storage in secretory granules, followed by secretion in response to glucose. Preproinsulin cDNA can be expressed in non-beta cells using cell-specific promoters (198-200). However, non-beta cells, unlike neuroendocrine cells, do not express the specific endopeptidases necessary to cleave proinsulin into mature insulin. To solve this problem, the cleavage site of the proinsulin can be modified so that it can be cleaved by the ubiquitous endoprotease furin (201,202). To reduce the complexity of having to engineer all of the complex machinery involved in glucose-responsive insulin secretion, cells such from the pituitary, adrenals, and liver, that possess at least some of the characteristics of beta cells, such as regulated secretion, have become the most studied candidates for insulin gene therapy.

8.1. Neuroendocrine Cells

Neuroendocrine cells such as those found in the pituitary and adrenals possess the secretory machinery needed for a regulated secretion of polypeptide hormones in response to external stimuli. Due to the expression of prohormone convertases (PC) 2 and 3, some of these cells can process proinsulin to insulin without any further genetic manipulation.

Many of the studies using neuroendocrine cells as a target for insulin gene therapy have been performed in AtT20, a neuroendocrine cell line derived from a mouse pituitary corticotroph tumor (198,203). AtT20 cells express PC2, PC3 (204) and glucokinase (205), but no GLUT-2. Transfection of the proinsulin gene allowed AtT20 cells to secrete insulin (206-209). Despite storing and secreting processed insulin, these cells did not exhibit glucose responsiveness (205,206,209). The expression of GLUT-2 in AtT20 cells was shown to establish glucose-responsive insulin secretion, but with insulin levels below the magnitude required physiologically (206,210). Moreover, diabetic animals transplanted with these cells developed Cushing's syndrome due to concomitant ACTH production (211). Recently, recombinant adenoviruses carrying GLUT2 and the islet isoform of glucokinase were delivered into intermediate lobe pituitary cells transfected with the insulin gene. This resulted in insulin secretion sufficient to cure diabetes in NOD mice (212). However, the insulin secretion was not glucose-responsive.

8.2. Hepatocytes

Hepatocytes express elements of the glucose-sensing machinery such as glucokinase and GLUT2, and respond to extracellular glucose levels. This property makes them an attractive target for gene therapy for diabetes. However, hepatocytes do not possess the regulated secretory pathway, and when the preproinsulin gene is expressed in them, exhibit constitutive secretion of proinsulin, with no mature insulin production (196,200). Efficient conversion of proinsulin to insulin was achieved with the introduction of the endoproteases PC2 and PC3

(213,214), and with the modification of the proinsulin cleavage sites into furin-cleavable sites (201,202,215).

Recombinant-adenovirus-mediated gene transfer of PDX-1 *in vivo* to the livers of BALB/C and C57BL/6 mice resulted in activation of the endogenous genes for mouse insulin 1 and 2, as well as the PC 1 and PC 3. This ameliorated hyperglycemia in streptozotocin-induced diabetic mice, although the subpopulation of liver cells that responded to this trans-differentiation is not fully characterized and may not even be hepatocytes (88).

Because glucose-induced insulin secretion through the regulated secretory pathway has been so difficult to reproduce in non-beta cells (177,196,215-217), there has been interest in using glucose-sensitive transcriptional response-elements coupled to constitutive insulin secretion to mimic beta cell function. Hepatocytes express a number of genes containing glucose-responsive promoters, including the phosphoenolpyruvate carboxykinase (PEPCK) and pyruvate kinase promoters (178,218,219). However, relying on transcriptional responses to control insulin secretion has a number of drawbacks, including delayed kinetics as well as complex responses to other factors that modulate transcription of the promoter, such as glucagon and cAMP in the case of the PEPCK promoter.

9. PERSPECTIVE

Cell-based therapy for diabetes has been the subject of intense interest over the past two decades. Its successful achievement would represent a cure for diabetes, with the end of the injections, glucose monitoring, and dietary restrictions required to maintain metabolic homeostasis and eliminate the extremely serious complications of the disease. Many years have been dedicated to the development of islet transplantation as a therapeutic option. The impressive results reported from University of Alberta, Canada (8) have brought a new light to the field of beta cell replacement. Unfortunately, the limited supply of primary tissue is still an obstacle to be overcome and is one of the most important targets of gene therapy for diabetes. The complexities involved in the beta cell machinery have made the engineering of non- beta cells an extremely difficult task. Despite some important achievements in the ex-vivo expansion of primary beta cells, our understanding of beta cell biology is not yet sufficient to move this approach into the clinic. The development of beta cell lines represent a very promising approach but has to overcome technical challenges and the potential risks of the growth stimulatory genes used in the process. Creating beta cells from pancreatic or other stem cells has great promise but is likely to require a greatly improved understanding of the process of beta cell growth and differentiation. Overall, the key to developing an effective beta cell replacement therapy for diabetes increasingly seems to rely upon a better understanding of the process of beta cell growth and development.

10. ACKNOWLEDGMENTS

11. REFERENCES

1. The Diabetes Control and Complications Trial Research Group: The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N. Engl. J. Med.* 329, 977-986 (1993)

2. Gough DA & J. C. Armour: Development of the implantable glucose sensor: What are the prospects and why is it taking so long? *Diabetes* 44, 1005-1009 (1995)

3. Tamada J A, N. J. V. Bohannon & R. O. Potts: Measurement of glucose in diabetic subjects using noninvasive transdermal extraction. *Nat. Med.* 1, 1198-1201 (1995)

4. Kost J, Mitragotri, S., Gabbay, R.A., Pishko, M., Langer, R.: Transdermal monitoring of glucose and other analytes using ultrasound. *Nat. Med.* 6, 347-350 (2000)

5. Ballinger WF & P. E. Lacy: Transplantation of intact pancreatic islets in rats. *Surgery* 72, 175-186 (1972)

6. Warnock GL, N. M. Kneteman, E. A. Ryan, A. Rabinovitch & R. V. Rajotte: Long-term follow-up after transplantation of insulin-producing pancreatic islets into patients with type 1 (insulin-dependent) diabetes mellitus. *Diabetologia* 35, 89-95 (1992)

7. Hering B J, M. D. Brendel, A. O. Schultz, B. Schultz, R. G. Bretzel & .: International islet transplant registry newsletter no. 7., Justus-Liebig-University of Giessen., Giessen, Germany: (1996).

8. Shapiro A, J. Lakey, E. Ryan, G. Korbutt, E. Toth, G. Warnock, N. Kneteman & R. Rajotte: Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N. Engl. J. Med.* 343, 230-238 (2000)

9. LaPorte RE M., Chang Y-F: Prevalence and incidence of insulin-dependent diabetes. In: Diabetes in America. Eds: Harris MI, CC, Stern MP, Boyko EJ, Reiber GE, Bennet PH, Washington, DC, (1995)

10. Hauptman PJ, C. K. O'Connor: Procurement and allocation of solid organ transplantation. *N. Engl. J. Med.* 327, 422-431 (1997)

11. Galili U, A. Tibell, B. Samuelsson, L. Rydberg & C. G. Groth: Increased anti-Gal activity in diabetic patients transplanted with fetal porcine islet cell clusters. *Transplantation* 59, 1549-1556 (1995)

12. McKenzie IF, M. Koulamanda, T. E. Mandel & M. S. Sandrin: Pig islets xenografs are susceptible to "anti-pig" but not Gal alpha (1,3) Gal antibody plus complement in Gal o/o mice. *J. Immunol.* 161 (1998)

13. Mirenda V L, B. LeMauf, A. Cassard, J..M. Huvelin, F. Boeffard, A. Faivre, J.P. Soulillou & I. Angeon: Intact pig pancreatic islet function in the presence of human xenoreactive natural antibody binding and complement activation. *Transplantation* 63, 1452-1462 (1997)

14. Karlsson-Parra A, A. Ridderstad, A. C. Wallgren, E. Möller, H. G. Ljunggren & O. Korsgren: Xenograft rejection of porcine islet-like cell clusters in normal and natural killer cell-depleted mice. *Transplantation* 61, 1313-1320 (1996)

15. van der Laan L J, C. Lockey, B. C. Griffeth, F. S. Frasier, C. A. Wilson, D. E. Onions, B. J. Hering, Z. Long, E. Otto, B. E. Torbett & D. R. Salomon: Infection by porcine endogenous retrovirus after islet xenotransplantation in SCID mice [see comments]. *Nature* 407, 90-94 (2000)

16. Levine F: Gene therapy for diabetes: strategies for beta cell modification and replacement. *Diabetes Metab. Rev.* 13, 209-246 (1997)

17. Levine F. & G. Leibowitz: Towards gene therapy of diabetes mellitus. *Mol. Med. Today* 5, 165-171 (1999)

18. Levine F, S. Wang, G. M. Beattie & A. Hayek: Human pancreatic cell lines: Developments and uses, The Regents of the University of California, USA (1998).

19. Groth C.G, O. Korsgren, A. Tibell, J. Tollemar, E. Moller, J. Bolinder, J. Ostman, F. P. Reinholt, C. Hellerstrom & A. Andersson: Transplantation of porcine fetal pancreas to diabetic patients. *Lancet* 344, 1402-1404 (1994)

20. Groth C G, A. Tibell, L. Wennberg & O. Korsgren: Xenoislet transplantation: experimental and clinical aspects. *J. Mol. Med.* 77, 153-154 (1999)

21. Otsu I, K. Dunleavy, C. J. P. Mullon, B. A. Solomon & A. P. Monaco: Treatment of diabetes by xenogeneic islets without immunosuppression: Use of a vascularized bioartificial pancreas. *Diabetes* 45, 342-347 (1996)

22. Sun Y, X. Ma, D. Zhou, I. Vacek & A. M. Sun: Normalization of diabetes in spontaneously diabetic cynomologus monkeys by xenografts of microencapsulated porcine islets without immunosuppression. *J. Clin. Invest.* 98, 1417-1422 (1996)

23. Onishi A, M. Iwamoto, T. Akita, S. Mikawa, K. Takeda, T. Awata, H. Hanada & A. C. Perry: Pig cloning by microinjection of fetal fibroblast nuclei [see comments]. *Science* 289, 1188-1190 (2000)

24. Bonner-Weir S, A. M. Davalli. , L. Scaglia, J. Hollister, G.C. Weir: Myths about the structure and function of porcine islets. *Xenotransplantation* 2, 207-212 (1996)

25. Weir GC, A. M. Davalli, Y Ogawa, L Scaglia, Y-J. Wu, J. Hollister J, S. Bonner-Weir : Transplantation of porcine islets in nude mice:implications for islet replacement therapy in humans. *Xenotransplantation* 2, 201-206 (1995)

26. Patience C, Y. Takeuchi & R. A. Weiss: Infection of human cells by an endogenous retrovirus of pigs. *Nat. Med.* 3, 282-286 (1997)

27. Weiss R A: Transgenic pigs and virus adaptation. *Nature* 391, 327-328 (1998)

28. Beattie G.M, V. Cirulli, A. D. Lopez & A. Hayek: Ex vivo expansion of human pancreatic endocrine cells. *J. Clin. Endocrinol. Metab.* 82, 1852-1856 (1997)

29. Beattie G M, P. Itkin-Ansari, V. Cirulli, G. Leibowitz, A. D. Lopez, S. Bossie, M. I. Mally, F. Levine & A. Hayek: Sustained proliferation of PDX-1+ cells derived from human islets. *Diabetes* 48, 1013-1019 (1999)

30. Beattie G M, J. S. Rubin, M. I. Mally, T. Otonkoski & A. Hayek: Regulation of proliferation and differentiation of human fetal pancreatic islet cells by extracellular matrix, hepatocyte growth factor, and cell-cell contact. *Diabetes* 45, 1223-1228 (1996)

31. Halvorsen T, G. Leibowitz & F. Levine: Telomerase activity is sufficient to allow transformed cells to escape from crisis. *Mol. Cell. Biol.* 19, 1864-1870 (1999)

32. Halvorsen T L, G. M. Beattie, A. D. Lopez, A. Hayek & F. Levine: Accelerated telomere shortening and senescence in human pancreatic islet cells stimulated to divide in vitro. *J. Endocrinol.* 166, 103-109 (2000)

33. Serrano M, A. W. Lin, M. E. McCurrach, D. Beach & S. W. Lowe: Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* 88, 593-602 (1997)

34. Otonkoski T, G. M. Beattie, M. I. Mally, C. Ricordi & A. Hayek: Nicotinamide is a potent inducer of endocrine differentiation in cultured human fetal pancreatic cells. *J. Clin. Invest.* 92, 1459-1466 (1993)

35. Otonkoski T, G. M. Beattie, J. S. Rubin, A. D. Lopez, A. Baird & A. Hayek: Hepatocyte growth factor/scatter factor has insulinotropic activity in human fetal pancreatic cells. *Diabetes* 43, 947-953 (1994)

36. Tuch BE, A. M. Simpson.: Experimental fetal islet transplantation. In: Pancreatic Islet Cell Transplantation. Eds: C, R. R.G. Landes, Pittsburgh, PA, 279-290, (1992)

37. Bonner-Weir S, L. A. Baxter, G. T. Schuppin & F. E. Smith: A second pathway for regeneration of adult exocrine and endocrine pancreas. A possible recapitulation of embryonic development. *Diabetes* 42, 1715-1720 (1993)

38. Vinik A, G. Pittenger, R. Rafaeloff & L. Rosenberg: Factors controlling pancreatic islet neogenesis. *Tumour Biol.* 14, 184-200 (1993)

39. Dudek R W, I. E. Lawrence, Jr., R. S. Hill & R. C. Johnson: Induction of islet cytodifferentiation by fetal mesenchyme in adult pancreatic ductal epithelium. *Diabetes* 40, 1041-1048 (1991)

40. Kerr-Conte J, F. Pattou, M. Lecomte-Houcke, Y. Xia, B. Boilly, C. Proye & J. Lefebvre: Ductal cyst formation in collagen-embedded adult human islet preparations. A means to the reproduction of nesidioblastosis in vitro. *Diabetes* 45, 1108-1114 (1996)

41. Bonner-Weir S, M. Taneja, G. C. Weir, K. Tatarkiewicz, K. H. Song, A. Sharma & J. J. O'Neil: In vitro cultivation of human islets from expanded ductal tissue. *Proc Natl Acad Sci U S A* 97, 7999-8004 (2000)

42. Klug M G, M. H. Soonpaa, G. Y. Koh & L. J.Field: Genetically selected cardiomyocytes from differentiating embrionic stem cells form stable intracardiac grafts. *J. Clin. Invest.* 98, 216-224 (1996)

43. Li ML, L. Pevny, R. Lovell-Badge & Smith A: Generation of purified neural precursors from embryonic stem cells by lineage selection. *Curr. Biol.* 8, 971-974 (1998)

44. Soria B, E. Roche, G. Berna, T. Leon-Quinto, R. J.A. & M. F.: Insulin-secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice. *Diabetes* 49, 157-162 (2000)

45. Ramiya V K, M. Maraist, K. E. Arfors, D. A. Schatz, A. B. Peck & J. G. Cornelius: Reversal of insulin-dependent diabetes using islets generated in vitro from pancreatic stem cells [see comments]. *Nat. Med.* 6, 278-282 (2000)

46. Wessels N K & J. H. Cohen.: Early pancreas organogenesis: morphogenesis, tissue interactions, and mass effects. *Dev. Biol.* 15, 237-270 (1967)

47. Kedinger M, P. Simon-Assmann., F. Bouziges, C. Arnold, E. Alexandre & K. Haffen: Smooth muscle actin expression during rat gut development and induction in fetal skin fibroblastic cells associated with intestinal embryonic ephitelium. *Differentiation* 43, 87-97 (1990)

48. Golosow N & C. Grobstein: Epitheliomesenchymal interaction in pancreatic morphogenesis. *Development* 4, 242-255 (1962)

49. Haffen K, K. Kedinger & P. Simon-Assmann.: Mesenchyme-dependent differentiation of epithelial progenitor cells in the gut. *J. Pediatr. Gastroenterol. Nutr.* 6, 14-23 (1987)

50. Hebrok M, S.K. Kim, & D.A. Melton: Notochord repression of endodermal Sonic hedgehog permits pancreas development. *Genes & Development* 12, 1705-1713 (1998)

51. Yamaoka T, C. Idehara, M. Yano, T. Matsushita, T. Yamada, S. Ii, M. Moritani, J. Hata, H. Sugino, S. Noji & M. Itakura: Hypoplasia of pancreatic islets in transgenic mice expressing activin receptor mutants. *J. Clin. Invest.* 102, 294-301 (1998)

52. Miralles F, P. Czernichow & R. Scharfmann: Follistatin regulates the relative proportions of endocrine versus exocrine tissue during pancreatic development. *Development* 125, 1017-1024 (1998)

53. Huotari M A, J. Palgi & T. Otonkoski: Growth factor-mediated proliferation and differentiation of insulin-producing INS-1 and RINm5F cells: identification of betacellulin as a novel beta-cell mitogen. *Endocrinology* 139, 1494-1499 (1998)

54. Seno M, H. Tada, M. Kosaka, R. Sasada, K. Igarashi,, Y. Shing, J. Folkman, M. Ueda & H. Yamada: Human betacellulin, a member of the EGF family dominantly expressed in pancreas and small intestine, is fully active in a monomeric form. *Growth Factors* 13, 181-191 (1996)

55. Miettinen P J, T. Otonkoski & Voutilainen: Insulin-like growth factor-II and transforming growth factor-alpha in developing human fetal pancreatic islets. *J. Endocrinol.* 138, 127-136 (1993)

56. Watada H, Y. Kajimoto, J. Miyagawa, T. Hanafusa, K. Hamaguchi, T. Matsuoka, K. Yamamoto, Y. Matsuzawa, R. Kawamori & Y. Yamasaki: PDX-1 induces insulin and glucokinase gene expressions in alphaTC1 clone 6 cells in the presence of betacellulin. *Diabetes* 45, 1826-1831 (1996)

57. Mashima H, H. Ohnishi, K. Wakabayashi, T. Mine, J. Miyagawa, T. Hanafusa, M. Seno, H. Yamada & I. Kojima: Betacellulin and activin A coordinately convert amylase-secreting pancreatic AR42J cells into insulin-secreting cells. *J. Clin. Invest.* 97, 1647-1654 (1996)

58. Mashima H, H. Shibata, T. Mine & I. Kojima: Formation of insulin-producing cells from pancreatic acinar AR42J cells by hepatocyte growth factor. *Endocrinology* 137, 3969-3976 (1996)

59. Demeterco C, G. M. Beattie, S. A. Dib, A. D. Lopez & A. Hayek: A Role for Activin A and Betacellulin in Human Fetal Pancreatic Cells Differentiation and Growth. *Journal of Endocrinology & Metabolism* 85, 3892-3897 (2000)

60. Sonnenberg E, D. Meyer, K. M. Weidner & C. Birchmeier: Scatter factor/hepatocyte growth factor and its receptor, the c-met tyrosine kinase, can mediate a signal exchange between mesenchyme and epithelia during mouse development. *J. Cell Biol.* 123, 223-235 (1993)

61. Otonkoski T, V. Cirulli, G. M. Beattie, M. I. Mally, G. Soto, J. S. Rubin & A. Hayek: A role for hepatocyte growth factor/scatter factor in fetal mesenchyme-induced pancreatic beta-cell growth. *Endocrinology* 137, 3131-3139 (1996)

62. Calvo E L, C. Boucher, G. Pelletier & J. Morisset: Ontogeny of hepatocyte growth factor and c-met/hgf receptor in rat pancreas. *Biochem. Biophys. Res. Commun.* 229, 257-263 (1996)

63. Garcia-Ocana A, K. K. Takane, M. A. Syed, W. M. Philbrick, R. C. Vasavada & A. F. Stewart: Hepatocyte Growth Factor Overexpression in the Islet of Transgenic Mice Increases Beta Cell Proliferation, Enhances Islet Mass, and Induces Mild Hypoglycemia. *J. Biol. Chem.* 275, 1226-1232 (2000)

64. Otonkoski T, M. I. Mally & A. Hayek: Opposite effects of beta-cell differentiation and growth on reg expression in human fetal pancreatic cells. *Diabetes* 43, 1164-1166 (1994)

65. Brelje T C, J. A. Parsons & R. L. Sorenson: Regulation of islet beta-cell proliferation by prolactin in rat islets. *Diabetes* 43, 263-273 (1994)

66. Zhou J, X. Wang, M. A. Pineyro & J. M. Egan: Glucagon-like peptide 1 and exendin-4 convert pancreatic AR42J cells into glucagon- and insulin-producing cells. *Diabetes* 48, 2358-2366 (1999)

67. Xu G, D. A. Stoffers, J. F. Habener & S. Bonner-Weir: Exendin-4 stimulates both beta-cell replication and neogenesis, resulting in increased beta-cell mass and improved glucose tolerance in diabetic rats. *Diabetes* 48, 2270-2276 (1999)

68. Dufayet de la Tour D, T. Halvorsen, C. Demeterco, B. Tyrberg, P. Itkin-Ansari, M. Loy, S.-J. Yoo, E. Hao, S. Bossie & F. Levine: beta-cell diferentiation from a human pancreatic cell line *in vitro* and *in vivo. Mol. Endocrinol. in press* (2000)

69. Edelman G M, Crossin, K.: Cell adhesion molecules: implication for a molecular histology. *Annu. Rev. Biochem.* 60, 155-190 (1991)

70. Takeichi M: Morphogenetic roles of classic cadherins. *Curr. Opin. Cell Biol.* 7, 619-627 (1995)

71. Dahl U, A. Sjødin & H. Semb: Cadherins regulate aggregation of pancreatic beta-cells in vivo. *Development* 122, 2895-2902 (1996)

72. Cirulli V, L. Crisa, G. M. Beattie, M. I. Mally, A. D. Lopez, A. Fannon, A. Ptasznik, L. Inverardi, C. Ricordi, T. Deerinck, M. Ellisman, R. A. Reisfeld & A. Hayek: KSA antigen Ep-CAM mediates cell-cell adhesion of pancreatic epithelial cells: morphoregulatory roles in pancreatic islet development. *J. Cell Biol.* 140, 1519-1534 (1998)

73. Vozzi C, S. Ullrich, A. Charollais, J. Philippe, L. Orci & P. Meda: Adequate connexin-mediated coupling is required for proper insulin production. *J. Cell Biol.* 131, 1561-1572 (1995)

74. Itkin-Ansari P, C. Demeterco, S. Bossie, D. Dufayet de la Tour, J. Movassat, M. Mally, G. Beattie, A. Hayek & F. Levine: PDX-1 and cell-cell contact act in synergy to promote delta-cell development in a human pancreatic endocrine precursor cell line. *Mol. Endocrinol.* 14, 814-822 (2000)

75. Ahlgren U, S. L. Pfaff, T. M. Jessell, T. Edlund & H. Edlund: Independent requirement fof ISL1 in formation of pancreatic mesenchyme and islet cells. *Nature* 385, 257-260 (1997)

76. Jonsson J, L. Carlsson, T. Edlund & H. Edlund: Insulin-promoter factor 1 is required for pancreas development in mice. *Nature* 371, 606-609 (1994)

77. Sander M & M. S. German: The beta cell transcription factors and development of the pancreas. *J. Mol. Med.* 75, 327-340 (1997)

78. Sosa-Pineda B, K. Chowdhury, M. Torres, G. Oliver & P. Gruss: The Pax4 gene is essential for differentiation of insulin-producing beta cells in the mammalian pancreas. *Nature* 386, 399-402 (1997)

79. St-Onge L, B. Sosa-Pineda, K. Chowdhury, A. Mansouri & P. Gruss: Pax6 is required for differentiation of glucagon-producing alpha-cells in mouse pancreas. *Nature* 387, 406-409 (1997)

80. Sussel L, J. Kalamaras, D. J. Hartigan-O'Connor, J. J. Meneses, R. A. Pedersen, J. L. Rubenstein & M. S. German: Mice lacking the homeodomain transcription factor Nkx2.2 have diabetes due to arrested differentiation of pancreatic beta cells. *Development* 125, 2213-2221 (1998)

81. Slack J M W.: Developmental biology of the pancreas. *Development* 121, 1569-1580 (1995)

82. Stoffers DA, N. T. Zinkin, V. Stanojevic, W. L.Clarke & J. F. Habener, J.F.: Pancreatic agenesis attributable to a single nucleotide delection in the human IPF1 gene coding sequence. *Nat. Genet.* 15, 106-110 (1997)

83. Ahlgren U, J. Jonsson & H. Edlund: The morphogenesis of the pancreatic mesenchyme is uncoupled from that of the pancreatic epithelium in IPF1/PDX1-deficient mice. *Development* 122, 1409-1416 (1996)

84. Offield M F, T. L. Jetton , P. A. Labosky, M. Ray, R. Stei, M. A. Magnuson, B. L. M. Hogan & Wright: PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. *Development* 122, 983-995 (1996)

85. Ohlsson H, K. Karlsson & T. Edlund: IPF1, a homeodomain-containing transactivator of the insulin gene. *EMBO J.* 12, 4251-4259 (1993)

86. Habener J F & D. A. Stoffers: A newly discovered role of transcription factors involved in pancreas development and the pathogenesis of diabetes mellitus. *Proc. Assoc. Am. Physicians* 110, 12-21 (1998)

87. Stoffers D A, Ferrer, J., Clarke, W.L., Habener, J.F.: Early-onset type II diabetes mellitus (MODY 4) linked to IPF1. *Nat. Genet.* 17, 138-139 (1997)

88. Ferber S, A. Halkin, H. Cohen, I. Ber, Y. Einav, I. Goldberg, I. Barshack, R. Seijffers, J. Kopolovic, N. Kaiser & A. Karasik: Pancreatic and duodenal homeobox gene 1 induces expression of insulin genes in liver and ameliorates streptozotocin-induced hyperglycemia [In Process Citation]. *Nat. Med.* 6, 568-572 (2000)

89. Stoppoloni G, D. Iafusco, B. M. Amodeo, E. De Felice, R. Toraldo, C. Betterle, L. D. Notarangelo, G. Tosi, R. S. Accolla & F. Prisco: A girl with diabetes and severe combined immunodeficiency from adenosine deaminase deficiency. *J. Pediatr. Endocrinol. Metab.* 10, 425-428 (1997)

90. Yamagata K, H. Furuta, N. Oda, P. J. Kaisaki, S. Menzel,, S. S. Fajans, S. Signorini, M. Stoffel & G. I. Bell.: Mutations in the hepatocyte nuclear factor-4a gene in maturity-onset diabetes of the young. *Nature* 384, 458-460 (1996)

91. Yamagata K, N. Oda, P. J. Kaisaki, S. Menzel, H. Furuta, M. Vaxillaire, L. Southam, R. D. Cox, G. M. Lathrop, V. V. Boriraj, X. Chen, N. J. Cox, Y. Oda, H. Yano, M. M. Le Beau, H. Nishigori, J. Takeda, S. S. Fajans, A. T. Hattersley, N. Iwasaki, T. Hansen, O. Pedersen, K. S. Polonsky, R. C. Turner, G. Velho, J.-C. Chevre, P. Froguel & G. I. Bell: Mutations in the hepatoycte nuclear factor-1alpha gene in maturity-onset diabetes of the young (MODY3). *Nature* 384, 455-460 (1996)

92. Sander M, A. Neubuser, J. Kalamaras, H. C. Ee, G. R. Martin & M. S. German: Genetic analysis reveals that PAX6 is required for normal transcription of pancreatic hormone genes and islet development. *Genes & Development* 11, 1662-1673 (1997)

93. Jensen J, P. Serup, C. Karlsen, T. Funder Nielsen & O. D. Madsen: mRNA profiling of rat islet tumors revelas Nkx 6.1 as a beta-cell-specific homeodomain transcription factor. *J. Biol. Chem.* 271, 18749-18758 (1996)

94. Oster A, J. Jensen, H. Edlund & L. I. Larsson: Homeobox gene product Nkx 6.1 immunoreactivity in nuclei of endocrine cells of rat and mouse stomach. *J. Histochem. Cytochem.* 46, 717-721 (1998)

95. Sander M, L. Sussel, J. Conners, D. Scheel, J. Kalamaras, F. D. Cruz, V. Schwitzgebel, A. Hayes-Jordan & M. S. German: Homeobox gene Nkx6.1 lies downstream of Nkx2.2 in the major pathway of beta-cell formation in the pancreas. *Development.* 127, 5533-5540 (2000)

96. Lee J E, S. M. Hollenberg, L. Snider, D. L. Turner, N. Lipnick & H. Weintraub: Conversion of Xenopus ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. *Science* 268, 836-843 (1995)

97. Naya F J, C. M. M. Stellrecht & M.-J. Tsai: Tissue-specific regulation of the insulin gene by a novel basic helix-loop-helix transcription factor. *Genes & Development* 9, 1009-1019 (1995)

98. Naya F J, H. P. Huang, Y. Qiu, H. Mutoh, F. J. DeMayo, A. B. Leiter & M. J. Tsai: Diabetes, defective

pancreatic morphogenesis, and abnormal enteroendocrine differentiation in BETA2/neuroD-deficient mice. *Genes & Development* 11, 2323-2334 (1997)

99. Hrabe de Angelis M, J. McIntyre & A. Gossler: Maintenance of somite borders in mice requires the Delta homologue Dll1. *Nature* 386, 717-721 (1997)

100. Oka C, T. Nakano, A. Wakehan, J. L. de la Pompa, C. Mori, T. Sakai, S. Okazaki, M. Kawaichi, K. Shiota, T. W. Mak & T. Honjo: Disruption of the mouse RPBJk gene. *Development* 121, 3291-3301 (1995)

101. Apelqvist A, H. Li, L. Sommer, P. Beatus, D. J. Anderson, T. Honjo, M. Hrabe de Angelis, U. Lendahl & H. Edlund: Notch signalling controls pancreatic cell differentiation. *Nature* 400, 877-881 (1999)

102. Sommer L, Q. Ma & D. J. Anderson: Neurogenins, a novel family of atonal-related bHLH transcriptional factors, are putative mammalian neuronal determination genes that reveal progenitor cell heterogeneity in the developing CNS and PNS. *Mol.Cell.Neurosci.* 8, 221-241 (1996)

103. Lardelli M, R. Willians, T. Mitsiadis & U. Lendahl: Expression of the Notch 3 intracellular domain in mouse central nervous system progenitor cells is lethal and leads to disturbed neural tube development. *Mech. Dev.* 59, 177-190 (1996)

104. Gradwohl G, A. Dierich, M. LeMeur & F. Guillemot: neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. *Proc. Natl. Acad. Sci. USA* 97, 1607-1611 (2000)

105. Sasai Y, R. Kageyama, Y. Tagawa, R. Shigemoto & S. Nakanishi: 2 mammalian helix loop helix factors structurally related to drosophila hairy and enhancer of split. *Genes Dev.* 6, 2620-2634 (1992)

106. Jensen J, E. E. Pedersen, P. Galante, J. Hald, R. S. Heller, M. Ishibashi, R. Kageyama, F. Guillemot, P. Serup & O. D. Madsen: Control of endodermal endocrine development by Hes-1. *Nat. Genet.* 24, 36-44 (2000)

107. Knaack D, D. M. Fiore, M. Surana, M. Leiser, M. Laurance, D. Fuscodemane, O. D. Hegre, N. Fleischer & S. Efrat: Clonal Insulinoma Cell Line That Stably Maintains Correct Glucose Responsiveness. *Diabetes* 43, 1413-1417 (1994)

108. Newgard C B: Cellular engineering and gene therapy strategies for insulin replacement in diabetes. *Diabetes* 43, 341-350 (1994)

109. Zhang Y, M. Warren-Perry, H. Sakura, J. Adelman, M. Stoffel, G. I. Bell, F. M. Ashcroft & R. C. Turner: No evidence for mutations in a putative beta-cell ATP-sensitive K+ channel subunit in MODY, NIDDM, or GDM. *Diabetes* 44, 597-600 (1995)

110. Asfari M, D. Janjic, P. Meda, G. Li, P. A. Halban & C. B. Wollheim: Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. *Endocrinology* 130, 167-178 (1992)

111. Poitout V, L. K. Olson & R. P. Robertson: Insulin-secreting cell lines: classification, characteristics and potential applications. *Diabetes & Metabolism* 22, 7-14 (1996)

112. Efrat S.: Genetic engineering of beta-cells for cell therapy of diabetes: cell growth, function, and immunogenicity. *Diabetes Reviews* 4, 224-234 (1996)

113. Rae P A, C. C. Yip & B. P. Schimmer: Isolation of cloned Syrian hamster insulinoma cell lines with limited capacity for insulin production. *Can. J. Physiol. Pharmacol.* 57, 819-824 (1979)

114. Chick W L, S. Warren, R. N. Chute, A. A. Like, V. Lauris & K. C. Kitchen: A transplantable insulinoma in the rat. *Proceedings of the National Academy of Sciences, USA* 74, 628-632 (1977)

115. Santerre R F, R. A. Cook, R. M. D. Crisel, J. D. Sharp, R. J. Schmidt, D. C. Williams & C. P. Wilson: Insulin synthesis in a clonal cell line of simian virus 40-transformed hamster pancreatic beta cells. *Proceedings of the National Academy of Sciences, USA* 78, 4339-4343 (1981)

116. Uchida S, S. Watanabe, T. Aizawa, A. Furuno & T. Muto: Polyoncogenicity and insulinoma-inducing ability of BK virus, a human papovarius, in Syrian golden hamsters. *J. Natl. Cancer Inst.* 63, 119-126 (1979)

117. Drucker D J: Molecular pathophysiology of glucagon-SV40 T antigen transgenic mice. *Am. J. Physiol.* 267, E629-E635 (1994)

118. Hanahan D: Heritable formation of pancreatic beta-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes. *Nature* 315, 33-40 (1985)

119. Radvanyi F, S. Christgau, S. Baekkeskov, C. Jolicoeur & D. Hanahan: Pancreatic beta cells cultured from individual preneoplastic foci, in a multistage tumorigenesis pathway: a potentially general technique for isolating physiologically representative cell lines. *Mol. Cell. Biol.* 13, 4223-4232 (1993)

120. Gazdar A F, W. L. Chick, H. K. Oie, H. L. Sims, D. L. King, G. C. Weir & V. Lauris: Continuous, clonal, insulin- and somatostatin-secreting cell lines established from a transplantable rat islet cell tumor. *Proceedings of the National Academy of Sciences, USA* 77, 3519-3523 (1980)

121. Madsen O D, L.-I. Larsson, J. F. Rehfeld, T. W. Schwartz, A. Lernmark, A. D. Labrecque & D. F. Steiner: Cloned cell lines from a transplantable islet cell tumor are heterogeneous and express cholecystokinin in addition to islet hormones. *J. Cell Biol.* 103, 2025-2034 (1986)

122. Carrington C A, E. D. Rubery, E. C. Pearson & C. N. Hales: Five new insulin-producing cell lines with differeng secretory properties. *J. Endocrinol.* 109, 193-200 (1986)

123. Eizirik D L, D. G. Pipeleers, Z. Ling, N. Welsh, C. Hellerstrom & A. Andersson: Major species differences between human and rodents in the susceptibility to pancreatic beta-cell injury. *Proceedings of the National Academy of Sciences, USA* 91, 9253-9256 (1994)

124. Sanvito F, P.-L. Herrera, J. Huarte, A. Nichols, R. Montesano, L. Orci & J.-D. Vassalli: TGF-beta1 influences the relative development of the exocrine and endocrine pancreas in vitro. *Development* 120, 3451-3462 (1994)

125. Hayek A, G. M. Beattie, V. Cirulli, A. D. Lopez, C. Ricordi & J. S. Rubin: Growth factor/matrix-induced proliferation of human adult beta-cells. *Diabetes* 44, 1458-1460 (1995)

126. Lund K, N. Blume, B. K. Michelsen, D. Bucchini & O. D. Madsen: Differential expression of non-allelic insulin genes in rodent islet tumour cells. *Journal of Molecular Ednocrinology* 11, 305-318 (1993)

127. De Vos A, H. Heimberg, E. Quartier, P. Huypens, L. Bouwens, D. Pipeleers & F. Schuit: Human and rat beta cells differ in glucose transporter but not in glucokinase gene expression. *J. Clin. Invest.* 96, 2489-2495 (1995)

128. Ferrer J, C. Benito & R. Gomis: Pancreatic islet GLUT2 glucose transporter mRNA and protein expression in humans with and without NIDDM. *Diabetes* 44, 1369-1374 (1995)

129. Soldevila G, M. Buscema, V. Marini, R. Sutton, R. F. L. James, S. R. Bloom, R. P. Robertson, R. Mirakian, R. Borrell-Pujol & G. F. Bottazzo: Transfection with SV40 gene of human pancreatic endocrine cells. *J. Autoimmun.* 4, 381-396 (1991)

130. Wang S, G. Beattie, M. Mally, V. Cirrulli, P. Itkin-Ansari, A. D. Lopez, A. Hayek & F. Levine: Isolation and characterization of a cell line from the epithelial cells of the human fetal pancreas. *Cell Transplant.* 6, 59-67 (1997)

131. Wang S, G. M. Beattie, M. I. Mally, V. Cirulli, A. D. Lopez, A. Hayek & F. Levine: Development and characterization of cell line from human pancreatic beta-cells and beta-cell precursors using retroviral vectors expressing SV40 T-antigen and H-rasval12. *Diabetes* 45 supplement 2, 285A (1996)

132. Gueli N, A. Toto, G. Palmieri, G. Carmenini, A. Delpino & U. Ferrini: In vitro growth of a cell line originated from a human insulinoma. *Journal of Experimental and Clinical Cancer Research* 6, 281-285 (1987)

133. Morgan R T, L. K. Woods, G. E. Moore, L. A. Quinn, L. McGavran & S. G. Gordon: Human cell line (COLO

357) of metastatic pancreatic adenocarcinoma. *Int. J. Cancer* 25, 591-598 (1980)

134. Pour P M, J. Permert, M. Mogaki, H. Fujii & K. Kazakoff: Endocrine aspects of exocrine cancer of the pancreas. *Am. J. Clin. Pathol.* 100, 223-230 (1993)

135. Vila M R, J. Lloreta, M. H. Schussler, G. Berrozpe, S. Welt & F. X. Real: New pancreas cancer cell lines that represent distinct stages of ductal differentiation. *Lab. Invest.* 72, 395-404 (1995)

136. Yamaguchi N, Y. Yamamura, K. Koyama, E. Ohtsuji, J. Imanishi & T. Ashihara: Characterization of new human pancreatic cancer cell lines which propagate in a protein-free chemically defined medium. *Cancer Res.* 50, 7008-7014 (1990)

137. Macfarlane W M, H. Cragg, H. M. Docherty, M. L. Read, R. F. James, A. Aynsley-Green & K. Docherty: Impaired expression of transcription factor IUF1 in a pancreatic beta-cell line derived from a patient with persistent hyperinsulinaemic hypoglycaemia of infancy (nesidioblastosis). *FEBS Lett.* 413, 304-308 (1997)

138. Macfarlane W M, R. E. O'Brien, P. D. Barnes, R. M. Shepherd, K. E. Cosgrove, K. J. Lindley, A. Aynsley-Green, R. F. James, K. Docherty & M. J. Dunne: Sulfonylurea receptor 1 and Kir6.2 expression in the novel human insulin-secreting cell line NES2Y. *Diabetes* 49, 953-960 (2000)

139. Lundqvist M & K. Oberg: In vitro culture of neuroendocrine tumors of the pancreas and gut. *Acta Oncol.* 28, 335-339 (1989)

140. Levine F, S. Wang, G. M. Beattie, M. I. Mally, V. Cirulli, A. D. Lopez & A. Hayek: Development of a cell line from the human fetal pancreas. *Transplant. Proc.* 27, 3410 (1995)

141. Wang S, G. M. Beattie, M. I. Mally, A. D. Lopez, A. Hayek & F. Levine: Analysis of a human fetal pancreatic islet cell line. *Transplant. Proc.* 29, 2219 (1997)

142. Levine F, S. Wang, G. M. Beattie, M. I. Mally, V. Cirulli, A. D. Lopez & A. Hayek: Properties of human pancreatic beta-cell lines developed using retroviral vectors expressing SV40 T antigen and H-ras-val12. *Diabetologia* 40 (Suppl 1), A118 (1997)

143. Bryan T M & R. R. Reddel: SV40-induced immortalization of human cells. *Crit. Rev. Oncog.* 5, 331-357 (1994)

144. Efrat S, M. Leiser, M. Surana, M. Tal, D. Fuscodemane & N. Fleischer: Murine Insulinoma Cell Line With Normal Glucose-Regulated Insulin Secretion. *Diabetes* 42, 901-907 (1993)

145. Efrat S, D. Fuscodemane, H. Lemberg, O. Alemran & X. R. Wang: Conditional transformation of a pancreatic

beta-cell line derived from transgenic mice expressing a tetracycline-regulated oncogene. *Proc. Natl. Acad. Sci. USA* 92, 3576-3580 (1995)

146. Gossen M & H. Bujard: Tight contol of gene expression in mammalian cells by tetracycline-responsive promoters. *Proceedings of the National Academy of Sciences, USA* 89, 5547-5551 (1992)

147. Wang S, G. M. Beattie, A. Hayek & F. Levine: Development of a VSV-G protein pseudotyped retroviral vector system expressing dominant oncogenes from a lacO-modified inducible LTR promoter. *Gene* 182, 145-150 (1996)

148. Zou Y.-R, W. Muller, H. Gu & K. Rajewsky: Cre-loxP-mediated gene replacement: a mouse strain producing humanized antibodies. *Curr. Biol.* 4, 1099-1103 (1994)

149. Metzger D, J. Clifford, H. Chiba & P. Chambon: Conditional site-specific recombination in mammalian cells using a ligand-dependent chimeric Cre recombinase. *Proceedings of the National Academy of Sciences, USA* 92, 6991-6995 (1995)

150. Kobayashi N, T. Fujiwara, K. A. Westerman, Y. Inoue, M. Sakaguchi, H. Noguchi, M. Miyazaki, J. Cai, N. Tanaka, I. J. Fox & P. Leboulch: Prevention of acute liver failure in rats with reversibly immortalized human hepatocytes [see comments]. *Science* 287, 1258-1262 (2000)

151. Rosengard A M, N. R. Cary, G. A. Langford, A. W. Tucker, J. Wallwork & D. J. White: Tissue expression of human complement inhibitor, decay-accelerating factor, in transgenic pigs. A potential approach for preventing xenograft rejection. *Transplantation* 59, 1325-1333 (1995)

152. Moolten F L: Drug sensitivity ("suicide") gene for selective cancer chemotherapy. *Cancer Gene Ther.* 1, 279-287 (1994)

153. Connors T A: The choice of prodrugs for gene directed enzyme prodrug therapy of cancer. *Gene Ther.* 2, 702-709 (1995)

154. Stone D, A. David, F. Bolognani, P. R. Lowenstein & M. G. Castro: Viral vectors for gene delivery and gene therapy within the endocrine system. *J. Endocrinol.* 164, 103-118 (2000)

155. Yang Y, F. A. Nunes, K. Berencsi, E. Gönczöl, J. F. Engelhardt & J. M. Wilson: Inactivation of E2a in recombinant adenoviruses improves the prospect for gene therapy in cystic fibrosis. *Nat. Genet.* 7, 362-369 (1994)

156. Miller D G, M. A. Adam & A. D. Miller: Gene transfer by retroviruses occurs only in cells that are actively replicating at the time of infection. *Mol. Cell. Biol.* 10, 4239-4242 (1992)

157. Blomer U, L. Naldini, T. Kafri, D. Trono, I. M. Verma & F. H. Gage: Highly efficient and sustained gene transfer in adult neurons with a lentivirus vector. *J. Virol.* 71, 6641-6649 (1997)

158. Naldini L, U. Blomer, P. Gallay, D. Ory, R. Mulligan, F. H. Gage, I. M. Verma & D. Trono: In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* 272, 263-267 (1996)

159. Castro M G, J. R. Davis, W. Xiong & P. R. Lowenstein: Recent developments in gene therapy: applications for the treatment of pituitary tumours. *Baillieres Best Pract Res Clin Endocrinol Metab* 13, 431-449 (1999)

160. Zhang R, D. Baunoch & L. J. DeGroot: Genetic immunotherapy for medullary thyroid carcinoma: destruction of tumors in mice by in vivo delivery of adenoviral vector transducing the murine interleukin-2 gene. *Thyroid* 8, 1137-1146 (1998)

161. Mirenda V, B. Charreau, J. Sigalla, A. Cassard, J. M. Huvelin, A. David, J. P. Soulillou, B. Le Mauff & I. Anegon: Xenoreactivity in the pig islet to human combination: feasibility of adenovirus-mediated gene transfer into pig islets. *Transplant. Proc.* 28, 808-810 (1996)

162. Becker T C, H. BeltrandelRio, R. J. Noel, J. H. Johnson & C. B. Newgard: Overexpression of hexokinase I in isolated islets of Langerhans via recombinant adenovirus. Enhancement of glucose metabolism and insulin secretion at basal but not stimulatory glucose levels. *J. Biol. Chem.* 269, 21234-21238 (1994)

163. Csete M E, P. Y. Benhamou, K. E. Drazan, O. Wu, D. F. McIntee, R. Afra, Y. Mullen, R. W. Busuttil & A. Shaked: Efficient gene transfer to pancreatic islets mediated by adenoviral vectors. *Transplantation* 59, 263-268 (1995)

164. Sigalla J, A. David, I. Anegon, M. Fiche, J. M. Huvelin, F. Boeffard, A. Cassard, J. P. Soulillou & B. Le Mauff: Adenovirus-mediated gene transfer into isolated mouse adult pancreatic islets: normal beta-cell function despite induction of an anti-adenovirus immune response. *Hum. Gene Ther.* 8, 1625-1634 (1997)

165. Leibowitz G, G. M. Beattie, T. Kafri, V. Cirulli, A. D. Lopez, A. Hayek & F. Levine: Gene transfer to human pancreatic endocrine cells using viral vectors. *Diabetes* 48, 745-753 (1999)

166. Muzzin P, R. C. Eisensmith, K. C. Copeland & S. L. Woo: Correction of obesity and diabetes in genetically obese mice by leptin gene therapy. *Proc. Natl. Acad. Sci. USA* 93, 14804-14808 (1996)

167. Wang M Y, K. Koyama, M. Shimabukuro, C. B. Newgard & R. H. Unger: OB-Rb gene transfer to leptin-resistant islets reverses diabetogenic phenotype. *Proc. Natl. Acad. Sci. USA* 95, 714-718 (1998)

168. Short D K, S. Okada, K. Yamauchi & J. E. Pessin: Adenovirus-mediated transfer of a modified human proinsulin gene reverses hyperglycemia in diabetic mice. *Am. J. Physiol.* 275, E748-756 (1998)

169. Raper S E & R. P. DeMatteo: Adenovirus-mediated in vivo gene transfer and expression in normal rat pancreas. *Pancreas* 12, 401-410 (1996)

170. Muruve D A, R. C. Manfro, T. B. Strom & T. A. Libermann: Ex vivo adenovirus-mediated gene delivery leads to long-term expression in pancreatic islet transplants. *Transplantation* 64, 542-546 (1997)

171. McClane S J, N. Chirmule, C. V. Burke & S. E. Raper: Characterization of the immune response after local delivery of recombinant adenovirus in murine pancreas and successful strategies for readministration. *Hum. Gene Ther.* 8, 2207-2216 (1997)

172. Anderson W F: Human gene therapy. *Nature* 392, 25-30 (1998)

173. Kochanek S, P. R. Clemens, K. Mitani, H. H. Chen, S. Chan & C. T. Caskey: A new adenoviral vector: Replacement of all viral coding sequences with 28 kb of DNA independently expressing both full-length dystrophin and beta-galactosidase. *Proc. Natl. Acad. Sci. USA* 93, 5731-5736 (1996)

174. Fisher K J, H. Choi, J. Burda, S. J. Chen & J. M. Wilson: Recombinant adenovirus deleted of all viral genes for gene therapy of cystic fibrosis. *Virology* 217, 11-22 (1996)

175. Burcin M M, G. Schiedner, S. Kochanek, S. Y. Tsai & B. W. O'Malley: Adenovirus-mediated regulable target gene expression in vivo. *Proc. Natl. Acad. Sci. USA* 96, 355-360 (1999)

176. Murphy J E, S. Zhou, K. Giese, L. T. Williams, J. A. Escobedo & V. J. Dwarki: Long-term correction of obesity and diabetes in genetically obese mice by a single intramuscular injection of recombinant adeno-associated virus encoding mouse leptin. *Proc. Natl. Acad. Sci. USA* 94, 13921-13926 (1997)

177. Sugiyama A, S. Hattori, S. Tanaka, F. Isoda, S. Kleopoulos, M. Rosenfeld, M. Kaplitt, H. Sekihara & C. Mobbs: Defective adenoassociated viral-mediated transfection of insulin gene by direct injection into liver parenchyma decreases blood glucose of diabetic mice. *Horm. Metab. Res.* 29, 599-603 (1997)

178. Lee H C, S. J. Kim, K. S. Kim, H. C. Shin & J. I. W. Yoon: Remission in models of type 1 diabetes by gene therapy using a single-chain insulin analogue. *Nature* 408, 483-488 (2000)

179. Breakefield X O & N. A. DeLuca: Herpes simplex virus for gene delivery to neurons. *New Biologist* 3, 474-485 (1991)

180. Johnson P A, A. Miyanohara, F. Levine, T. Cahill & T. Friedmann: Cytotoxicity of a replication-defective mutant of herpes simplex virus type 1. *J. Virol.* 66, 2952-2965 (1992)

181. Frenkel N, O. Singer & A. D. Kwong: Minireview: the herpes simplex virus amplicon--a versatile defective virus vector. *Gene Ther.* 1 Suppl 1, S40-46 (1994)

182. Liu Y, A. Rabinovitch, W. Suarez-Pinzon, B. Muhkerjee, M. Brownlee, D. Edelstein & H. J. Federoff: Expression of the bcl-2 gene from a defective HSV-1 amplicon vector protects pancreatic beta-cells from apoptosis. *Hum. Gene Ther.* 7, 1719-1726 (1996)

183. Weiss R A & C. S. Tailor: Retrovirus receptors. *Cell* 82, 531-533 (1995)

184. Bordignon C, L. D. Notarangelo, N. Nobili, G. Ferrari, G. Casorati, P. Panina, E. Mazzolari, D. Maggioni, C. Rossi, P. Servida & et al.: Gene therapy in peripheral blood lymphocytes and bone marrow for ADA- immunodeficient patients. *Science* 270, 470-475 (1995)

185. Wolf D, C. Richter-Landsberg, M. P. Short, C. Cepko & X. O. Breakefield: Retrovirus-mediated gene transfer of beta-nerve growth factor into mouse pituitary line AtT-20. *Mol Biol Med* 5, 43-59 (1988)

186. O'Malley B W, Jr. & F. D. Ledley: DNA- and viral-mediated gene transfer in follicular cells: progress toward gene therapy of the thyroid. *Laryngoscope* 103, 1084-1092 (1993)

187. O'Malley B W, Jr., R. M. Adams, M. L. Sikes, T. Sawada & F. D. Ledley: Retrovirus-mediated gene transfer into canine thyroid using an ex vivo strategy. *Hum. Gene Ther.* 4, 171-178 (1993)

188. Zufferey R, D. Nagy, R. J. Mandel, L. Naldini & D. Trono: Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nat. Biotechnol.* 15, 871-875 (1997)

189. Ju Q, D. Edelstein, M. D. Brendel, D. Brandhorst, H. Brandhorst, R. G. Bretzel & M. Brownlee: Transduction of non-dividing adult human pancreatic beta cells by an integrating lentiviral vector. *Diabetologia* 41, 736-739 (1998)

190. Giannoukakis N, Z. Mi, A. Gambotto, A. Eramo, C. Ricordi, M. Trucco & P. Robbins: Infection of intact human islets by a lentiviral vector. *Gene Ther.* 6, 1545-1551 (1999)

191. Mulligan R C: The basic science of gene therapy. *Science* 260, 926-931 (1993)

192. Saldeen J, D. T. Curiel, D. L. Eizirik, A. Andersson, E. Strandell, K. Buschard & N. Welsh: Efficient gene transfer to dispersed human pancreatic islet cells in vitro using adenovirus-polylysine/DNA complexes or polycationic liposomes. *Diabetes* 45, 1197-1203 (1996)

193. Saldeen J, S. Sandler, K. Bendtzen & N. Welsh: Liposome-mediated transfer of IL-1 receptor antagonist gene to dispersed islet cells does not prevent recurrence of disease in syngeneically transplanted NOD mice. *Cytokine* 12, 405-408 (2000)

194. Bartlett R J, S. L. Secore, M. Denis, L. Fernandez, A. Tzakis, R. Alejandro & C. Ricordi: Toward the biologic

release of human insulin from skeletal muscle. *Transplant. Proc.* 29, 2199-2200 (1997)

195. Goldfine I D, M. S. German, H. C. Tseng, J. Wang, J. L. Bolaffi, J. W. Chen, D. C. Olson & S. S. Rothman: The endocrine secretion of human insulin and growth hormone by exocrine glands of the gastrointestinal tract. *Nat. Biotechnol.* 15, 1378-1382 (1997)

196. Kolodka T M, M. Finegold, L. Moss & S. L. Woo: Gene therapy for diabetes mellitus in rats by hepatic expression of insulin. *Proc. Natl. Acad. Sci. USA* 92, 3293-3297 (1995)

197. Taniguchi H, H. Nakauchi, H. Iwata, H. Amemiya & K. Fukao: Treatment of diabetic mice with encapsulated fibroblasts producing human proinsulin. *Transplant. Proc.* 24, 2977-2978 (1992)

198. Moore H-P, M. D. Walker, F. Lee & R. B. Kelly: Expressing a human proinsulin cDNA in a mouse ACTH-secreting cell. Intracellular storage, proteolytic processing, and secretion on stimulation. *Cell* 35, 531-538 (1983)

199. Simpson A M, B. E. Tuch, M. A. Swan, J. Tu & G. M. Marshall: Functional expression of the human insulin gene in a human hepatoma cell line (HEP G2). *Gene Ther.* 2, 223-231 (1995)

200. Vollenweider F, J.-C. Irminger, D. J. Gross, L. Villa-Komaroff & P. A. Halban: Processing of proinsulin by transfected hepatoma (FAO) cells. *J. Biol. Chem.* 267, 14629-14636 (1992)

201. Groskreutz D J, M. X. Sliwkowski & M. X. Gorman: Genetically engineered proinsulin constitutively processed and secreted as mature, active insulin. *J. Biol. Chem.* 269, 6241-6245 (1994)

202. Falqui L, S. Martinenghi, C. Berra, L. Monti, B. E. Leone, G. Pozza & C. Bordignon: Human proinsulin production in primary rat hepatocytes after retroviral vector gene transfer. *J. Mol. Med.* 77, 250-253 (1999)

203. Furth J, E. L. Gadsen & A. C. Upton: ACTH secreting transplantable pituitary tumors. *Proc. Soc. Exp. Biol. Med.* 84, 253-254 (1953)

204. Vindrola O & I. Lindberg: Biosynthesis of the prohormone convertase mPC1 in AtT-20 cells. *Mol. Endocrinol.* 6, 1088-1094 (1992)

205. Hughes S D, C. Quaade, J. L. Milburn, L. C. Cassidy & C. B. Newgard: Expression of normal and novel glucokinase mRNAs in anterior pituitary and islet cells. *J. Biol. Chem.* 266, 4521-4530 (1991)

206. Hughes S D, J. H. Johnson, C. Quaade & C. B. Newgard: Engineering of glucose-stimulated insulin secretion and biosynthesis in non-islet cells. *Proceedings of the National Academy of Sciences, USA* 89, 688-692 (1992)

207. Irminger J C, F. M. Vollenweider & M. Neerman-Arbez: Human proinsulin conversion in the regulated and the constitutive pathway of transfected AtT20 cells. *J. Biol. Chem.* 269, 1756-1762 (1994)

208. Kaufmann J E, J. C. Irminger & P. A. Halban: sequence requirements for proinsulin processing at the B-chain/C-peptide junction. *Biochem. J.* 310, 869-874 (1995)

209. Stewart C, N. A. Taylor, K. Docherty & C. J. Bailey: Insulin delivery by somatic cell gene therapy. *J Mol Ednocrinol* 11, 335-341 (1993)

210. Hughes S D, C. Quaade, J. H. Johnson, S. Ferber & C. B. Newgard: Transfection of AtT-20ins cells with GLUT-2 but not GLUT-1 confers glucose-stimulated insulin secretion. *J. Biol. Chem.* 268, 15205-15212 (1993)

211. BeltrandelRio H, W. J. Schnedl, S. Ferber & C. B. Newgard: Genetic engineering of insulin secreting cell lines. In: Pancreatic Islet Transplantation. Eds: Lanza, RP & Chick, WL. Landes, Austin, TX, 1, 169-178, (1994)

212. Lipes M A, R. Faradji, E. Havari & R. C. Mulligan: Cellular engineering approaches to the treatment of IDDM. *Diabetes*, A244 (1999)

213. Kaufmann J E, J.-C. Irminger, J. Mungall & P. A. Halban: Proinsulin conversion in GH3 cells after coexpression of human proinsulin with the endoproteases PC2 and/orPC3. *Diabetes* 46, 978-982 (1997)

214. Vollenweider F, J. Kaufmann, J.-C. Irminger & P. A. Halban: Processing of proinsulin by furin,PC2, and PC3 in (co)transfected COS (monkey kidney) cells. *Diabetes* 44, 1075-1080 (1995)

215. Muzzin P, R. C. Eisensmith, K. C. Copeland & S. L. Woo: Hepatic insulin gene expression as treatment for type 1 diabetes mellitus in rats. *Mol. Endocrinol.* 11, 833-837 (1997)

216. Valera A, C. Fillat, C. Costa, J. Sabater, J. Visa, A. Pujol & F. Bosch: Regulated expression of human insulin in the liver of transgenic mice corrects diabetic alterations. *FASEB J.* 8, 440-447 (1994)

217. Selden R F, M. J. Skoskiewicz, P. S. Russell & H. M. Goodman: Regulation of insulin gene expression: Implications for gene therapy. *N. Engl. J. Med.* 317, 1067-1076 (1987)

218. Gros L, L. Montoliu, E. Riu, L. Lebrigand & F. Bosch: Regulated production of mature insulin by non-beta-cells. *Hum. Gene Ther.* 8, 2249-2259 (1997)

219. Thule P M, J. Liu & L. S. Phillips: Glucose regulated production of human insulin in rat hepatocytes. *Gene Ther.* 7, 205-214 (2000)

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