International Max Planck Research School for Molecular Cell biology and Bioengineering

The Vascular Basement Membrane: A Niche for Insulin Gene Expression And Beta Cell Proliferation

Dissertation

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Contents

SUMMARY

Endocrine pancreatic beta cells depend on endothelial signals for their differentiation and function. In the embryo, differentiation and delamination of insulin-producing beta cells from pancreatic epithelium strictly require endothelial cells. During later embryonic development, delaminated beta cells aggregate to form islets. These cell aggregates express high levels of the vascular endothelial growth factor VEGF-A to attract VEGFR2-expressing endothelial cells, which form a vascular network within the islets. This particularly dense vascular network is required for proper β-cell function and islet size. The insulin, released from β-cells into the bloodstream, lowers blood glucose levels. Any deficiencies in insulin production and secretion, due to β-cell disfunction and/or destruction, cause hyperglycemia and diabetes, the most common metabolic disorder in humans. Islet transplantation is one of the most promising approaches to cure diabetes. During islet isolation procedure there is a partial removal of intraislet endothelial cells that contributes to transplant failure. All these findings raise the question of whether endothelial cells create a permissive environment, or a vascular niche for beta cell function and growth.

The study described here focuses on understanding the molecular basis of the interaction between the β-cells and endothelial cells in the pancreatic islets. My work shows that β-cells, in contrast to the exocrine pancreatic cells do not form a basement membrane. Instead, by using VEGF-A, they attract endothelial cells, which form capillaries with a vascular basement membrane next to β-cells. I have identified laminins, among other vascular basement membrane proteins, as endothelial signals, which promote insulin gene expression and proliferation in beta cells. I further demonstrate that β1-integrin is required for the β-cell response to the laminins. The proposed mechanism explains why β-cells must interact with endothelial cells and it may therefore lead to the development of new strategies for the treatment of diabetes.

INTRODUCTION

1. Pancreatic beta cells

The pancreatic islets of Langerhans[1] (Morrison, 1937), are endocrine glands made by the aggregation of 4 major secretory cell types. They represent approximately 2% of the total pancreatic mass. Each islet (Figure1) is composed of 2000-4000 cells, ~60%-80% of which secrete insulin and are referred to as beta cells. Glucagon secreting alpha-cells account for ~ 15-20% of the islet cells, while somatostatin-secreting delta-cells, and pancreatic polypeptide-secreting cells (PP) account ~ 5-10%, and < 2% of the total islet cell number respectively. Islets are generally oval in shape and their core consists primarily of beta cells, while non-beta- cells are located mostly in the surrounding mantle. Like all endocrine glands, islets secrete their hormones into the bloodstream and are highly vascularised. The specific role of beta cells[2] is to monitor glucose levels in the blood and to secrete the hormone insulin[3] when glucose concentration is > 5.5 mM. Insulin in turn, lowers glucose levels by activating insulin receptors and thereby increasing the permeability of cells to glucose.

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[1]Named after the German anatomist Paul Langerhans (1847- 1888) who discovered them 1869 in Berlin.

[2] The first evidence of the functional significance of the pancreas for regulation of glucose levels was obtained 1889 by Mering and Minkowski who found that pancreatectomy in dogs leads to diabetes.

[3] Insulin was discovered in 1922 by Frederick Banting and Charles Best using pancreatic extracts as a source of the hormone to treat diabetes in pancreatectomized dogs (Banting, F.G., and C.H. Best. 1990. Pancreatic extracts. 1922. *J Lab Clin Med*. 115:254-72)

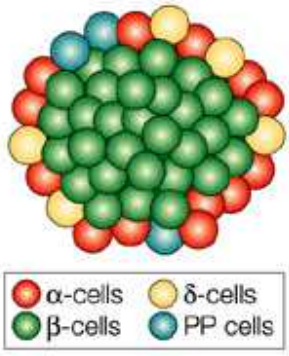

Figure 1. **Schematic representation of an islet of Langerhans.** Each islet of Langerhans consists of four hormone-producing cell types: alpha-, beta-, delta- and pancreatic polypeptide (PP) cells. Alpha-cells (red) secrete glucagon and make up 15–20% of the endocrine pancreas. Beta-cells (green) secrete insulin and make up 60–80% of the endocrine pancreas. Delta-cells (yellow) secrete somatostatin and make up 5–10% of the endocrine pancreas, whereas PP cells (blue) secrete PP and make up less than 2% of the endocrine pancreas (from Nat Rev Genet. 2002 Jul; 3(7): 524-32, Edlund H.).

1.1. Insulin gene expression

Insulin was discovered in 1922 by Banting and Best. In most species it exists as a single gene, whereas in the mouse and the rat two non-allelic insulin genes are present.

The human insulin gene is located on the short arm of chromosome 11 (Harper et al., 1981), the rat insulin 1 and 2 genes are colocalized on chromosome 1(Soares et al., 1985) and, in the mouse genes, are positioned on two different chromosomes, insulin 1 on chromosome 19 (Davies et al., 1994) and insulin 2 on chromosome 7 (Duvillie et al., 1998).

The rodent insulin 2 and the human insulin gene contain three exons and two introns, whilst insulin 1 lacks the second intron (Melloul et al., 2002).

Insulin is synthesized as a single precursor molecule, preproinsulin, which is posttranslationally modified to give the mature hormone (Wentworth et. al., 1986).

Analysis of the gene and polypeptide sequences of the two rodent (pre)proinsulins has revealed closely homologous genes, differing only by six amino acids and a deletion of two

amino acids in the C-peptide of the ins-1 protein. In adult islets, both genes appear to be coordinately expressed and regulated (Melloul et al, 2002). Early studies suggested that the two insulins are present in approximately equal amounts (Markussen, 1971; Steiner et al., 1969), and this observation was supported by later studies examining (pre)proinsulin mRNA transcripts in mice (Wentworth et. al., 1986 et. al., 1986; Koranyi et al., 1989; Permut et al., 1972) and protein levels (Permut et al., 1972) in rats. In contrast, other studies presented evidence suggesting that either ins1 (Villa-Komaroff et al., 1992) or ins2 is the predominantly expressed gene in mice (Leroux et al., 2001, and Linde et al., 1989).

It is often assumed that both genes are subject to the same regulatory pathways, given their structural similarity. A few studies, however, have suggested that differential control of gene expression may occur in some circumstances resulting in a shift in the ratio of ins1 to ins2 expression (Roderigo-Milne et al., 2002).

1.1.1. First embryonic insulin gene expression in mouse

In the developing mouse embryo, both mRNAs could be detected at day 9.5 of gestation when the dorsal pancreatic anlage appears. At days 9.5 and 10.5, when the ventral anlage appears, there are fewer proinsulin 2 mRNAs than proinsulin 1 mRNAs. During embryonic development the relative expression of the two genes changes several times, suggesting some independence in their regulatory mechanisms. (Deltour et al., 1993).

1.1.2. Transcriptional regulation of insulin gene expression

Expression of the insulin gene in adults is restricted to the β-cells of the islets of Langerhans (Marshak et al., 2001). This restriction of insulin production derives primarily from

constraints imposed at the level of transcription of the gene encoding preproinsulin (the *insulin* gene), rather then at the level of translation of the nascent mRNA (Iype et al., 2005).

The most important regulator of insulin gene expression is glucose. This regulation is a complex process that includes the activation of transcriptional and posttranscriptional mechanisms (Permutt and Kipnis, 1972). Posttranscriptional mechanisms account for the enhanced biosynthesis of insulin (Ito and Okamoto, 1980; Welsh et al., 1986a; Welsh et al., 1986 b). It was recently found that glucose entry is followed very rapidly by the nucleocytoplasmic translocation of a multifunctional mRNA binding protein termed polypyrimidine tract-binding protein (PTB). Once in the cytosol, PTB binds and stabilizes premade mRNA encoding insulin and other proteins of the secretory granules (Knoch et al., 2004).

Glucose induction of insulin transcription is a slow process that requires several hours before being activated. Previous research has led to the identification of various conserved regulatory sequence motifs within the insulin promoter region (German et al., 1995) and to the isolation of transcription factors that activate insulin gene expression. Among these motifs, E, A, and C1/RIPE3b seem to play a major role in the β-cell specific insulin gene expression.

E-boxes. Studies on rat insulin promoter I led to the identification of two closely related repeats, which play an important role in beta cell-specific expression. The core sequence of these elements belongs to the class of regulatory motifs designated E boxes, whose shared consensus sequence is: CANNTG. The E boxes bind proteins of the bHLH (basic helix-loop-helix) family, which function as potent transcriptional activators. E1 element, in particular, binds the heterodimeric complex IEF1 (reviewed in Massari and Murre, 2000). This complex,

in turn, interacts with the cell type restricted bHLH protein BETA2/NeuroD, an important regulator of insulin gene expression (Naya et al., 1995).

A-boxes. Regulatory elements that contain AT rich sequences are defined as A boxes (A1-A5) (German et al., 1995), and with the exception of A2 (GGAAAT), they all have the core TAAT sequence. This motif is recognized by factors of the homeodomain-containing protein family. A well-known transcription factor of this family is the pancreatic duodenal homeobox-1 (PDX-1) (described below).

RIPE3b. The positive control of the rat insulin II enhancer (RIPE3) is mediated by the RIPE3b and E1/ICE elements. RIPE3b is composed of two overlapping sequences, termed A2 and C1 elements. A2 binds three specific complexes, of which only A2.2 is expressed in insulin producing cell lines (Harrington and Sharma, 2001). C1 binds two elements, the β-cell specific RIPE3b-Activator/C1, and the widely distributed RIPE3b2 (Shieh and Tsai, 1991). RIPE 3b1, in turn, is composed of several proteins, and its binding activity depends on tyrosine-phosphorylation, with reports suggesting that tyrosine phosphatase or kinase signaling modulate the expression of insulin by acting upon RIPE3b.

Other elements involved in the positive regulation of insulin gene expression are cyclic AMP response element (CRE), mainly through the protein kinase A-dependent phosphorylation of the transcription factor CREB (Daniel et al., 1998), the G1 or GAGA box, and the insulin linked polymorphic region (ILPR), most likely via the transcription factor Pur-1 (Kennedy and Rutter, 1992).

The JAK/STAT pathway is also involved in the transcription of insulin, as a **STAT binding element** is present in the rat insulin I promotor. STAT proteins form homodimeric and

heterodimeric DNA binding complexes following their tyrosine phosphorylation by JAK in response to stimulation of β-cells by growth hormone (GH) and prolactin (PRL) (Herrington et al., 2000).

In addition to the described conserved regulatory sequence motifs, which are necessary for β-cell specific insulin gene expression, it is tempting to speculate there might be also other type of sequence elements, which are involved in the modulation of insulin expression. This assumption is based on the finding of Bosco et al. that glucose stimulated insulin secretion in-vitro is improved when primary beta cells were cultured on laminin-5 rich matrix (Bosco et al., 2000). Although the last study did not show the molecular mechanism explaining insulin secretion modulation in response to matrix components, it could be expected that this response reflect changes on a transcriptional level. The identification of genes that are dependent upon the presence of the ECM for their transcription led to the discovery of **'ECM response elements'** within gene promoters (Boudreau et al., 1995). The extracellular matrix (ECM) has been shown to play an important role in development and tissue specific gene expression, yet the mechanism by which genes receive signals from ECM is poorly understood (Seid et al., 1997). An ECM-response element was first identified in mammary epithelial cells in the promoter of the β-casein gene (Schmidhauser et al., 1992). The 161 bp β-casein enhancer element (BCE-1) drives transcription in an ECM and prolactin-dependent manner when attached to the truncated and otherwise inactive 121 bp promoter of the β-casein gene. Expression of the albumin gene in hepatocytes is also dependent on ECM. The considerably larger enhancer located in the albumin gene promoter is activated in the presence of the appropriate ECM and thus can also be considered to be an ECM-response element (Boudreau et al., 1995).

It is not known whether there are any ECM response elements within the insulin gene promoter region. However, it is quite possible for this to be the case since according to the

results in the study I did it is clear that basement membrane proteins, in particular laminins, influence insulin gene transcription. In addition, according to a recent study (Hammar et al., 2005), according to which 804-G-ECM (laminin-5 rich ECM derived from 804G cells) induces transient translocation and DNA-binding activity of the p65 subunit of NF-kB in primary pancreatic beta cells, which leads to improved glucose stimulate insulin secretion.

Regulated insulin gene transcription relies on the interaction of the sequence motifs in the promoter with a number of ubiquitous and islet specific transcription factors. These interactions determine the positive and negative regulation of insulin gene expression and its inducibility by physiological stimuli. Transcription factors play an important role during pancreatic development ensuring normal differentiation of the islet endocrine cells. Among these factors are Pdx1, Hlxb9, Isl1, Hes1, Ngn3, NeuroD1/BETA2, Pax4, Pax6, Nkx2.2 andNkx6.1 and Maf A (Habener et al., 2005). In mature adult β-cells, expression of specific factors such as Pdx1, Hnf-1α, NeuroD1/BETA2, MafA is essential in maintaining normal β-cell function, including the regulation of insulin gene transcription. (Habener et al., 2004; Kaneto et al., 2005).

Pdx-1. Pancreatic duodenal homeobox-1 (also named IPF1 in humans other aliases include IDX1, STF1, and Iuf1) which is expressed in adult β-cells, is a major transactivator of the insulin gene (Ohlsson et al., 1993; Marshak et al., 1996), and of other islet specific genes, such as GLUT2 (Waeber et al., 1996), glucokinase (Watada et al., 1996a; Watada et al., 1996b), IAPP (Carty at al., 1997) and somatostatin (Leonard et al., 1993).

Pdx-1 first appears in the digestive tract of the mouse at embryonic day (e) 8.5, a day before the dorsal bud of the pancreas develops. Pdx-1 is considered to be the master regulator of pancreatic development and β-cell differentiation. It has a dual role as an inducer of the endocrine lineage from ductal epithelial cells and in the maturation of β-cells (Hill and Duvillie, 2000). Pdx-1 may be directly activated by the transcription factors NeuroD1

(Sharma et al., 1997), Hnf-1α, and Hnf-3β (Ben-Shushan et al., 2001). Lowered Pdx-1 expression or activity resulting in impaired expression of both GLUT-2 and insulin could cause hyperglycemia, which may progress to Type 2 diabetes (described below). Pdx-1 expression is required in mature β-cells to maintain hormone production, GLUT-2 expression, and euglycemia (Ahlgren et al., 1998).

NeuroD1/BETA2. Neurogenic differentiation 1 or β-cell E box transactivator 2 is a cell-type restricted basic helix–loop–helix (bHLH) transcription factor expressed in all endocrine cells (Naya et al., 1995). It is expressed in the mouse pancreatic bud at e9.5.

NeuroD1/Beta2 heterodimerizes with ubiquitous bHLH proteins of the E2A family to regulate the transcription of the insulin gene. Mice homozygous for a targeted disruption of the neuroD1/BETA2 gene survive to birth, but die within three to five days postpartum of severe hyperglycemia. The islets of these mice are dysmorphic and have markedly diminished number of endocrine cells arranged in streaks and irregular aggregates and reduced number of β-cells (Habener at al., 2005).

Hnf-1α. The hepatocyte nuclear factor, Hnf-1α, is expressed at e13.5 in the developing mouse pancreas and in β-cells during adulthood. This transcription factor has been proposed to regulate the gene expression of insulin and the glucose transporter, GLUT-2 (Cha et al., 2000; Wang et al., 1998). It also appears to be essential for maintaining insulin storage (Wang et al., 1998). In mice, defects in the Hnf-1α gene result in the development of Type 2 diabetes (described below) 2 weeks after birth. Null mutant mice for Hnf-1α have impaired glucose stimulated insulin secretion (Dukes et al., 1998).

MafA. A recently isolated pancreatic β-cell-specific transcription factor, which is a potent activator of insulin gene transcription. MafA, a basic leucine zipper transcription factor

(Kataoka et al., 2002), controls β-cell-specific expression of the insulin gene through a *cis*-regulatory element RIPE3b and functions as a potent transactivator for the insulin gene (Matsuoka et al., 2004; Kataoka et al., 2004). During pancreas development, MafA expression is first detected at the beginning of the principal phase of insulin-producing cell production (Matsuoka et al., 2004). In addition, while both PDX-1 and NeuroD are expressed in various types of cells in islets, MafA is the only β-cell-specific transactivator for the insulin gene. MafA DNA binding activity is reduced under diabetic conditions in parallel with the decrease of insulin gene expression (Sharma et al., 1994). Recent studies showed that MafA expression in the liver, together with PDX-1 and NeuroD, markedly induces insulin gene transcription and dramatically ameliorates glucose tolerance in diabetic model animals (Kaneto et al., 2005; Kataoka et al., 2002). These results suggest a crucial role of MafA as a novel therapeutic target for diabetes and imply that a combined therapy in terms of manipulating MafA, Pdx-1 and NeuroD should be useful for replacing the reduced β-cell function found in diabetes.

1.2. Beta cell proliferation

1.2.1. Self-replication versus ductal stem cell differentiation

During development, β-cells are generated from a population of pancreatic $Ngn3^{+}$ progenitor cells (Edlund, 2002; Wilson et al., 2003).

In the neonatal period new β cells are formed by replication of differentiated β cells, which results in a massive increase in β cell mass (Finegood et al., 1995; Svenstrup et al., 2002).

The literature on pancreatic beta cells and islets of Langerhans contains studies suggesting various mechanisms for beta-cell homeostasis and regenerative repair in the adult. In general, formation of new beta cells can take place by two pathways: neogenesis from putative progenitor stem cells (Nielsen et al., 1999) or replication of already differentiated beta cells

(Dor et al., 2004). Some immunohistochemical observations, suggesting a stem-cell origin for beta-cells (Bonner-Weir& Sharma, 2002) led to the proposal that these adult pancreatic stem or progenitor cells reside in the epithelium of pancreatic ducts (Bonner Weir et al., 1993; Zajicek et al., 1990), inside islets (Zulewski et al., 2001) or in the bone marrow (Ianus et al., 2003). Others have suggested that beta cells form in the adult by transdifferentiation of pancreatic acinar cells (Lipsett et al., 2002), islet cells that express hormones other than insulin (Guz et al., 2001), or splenocytes (Kodama et al., 2003). However, according to Dor (Dor et al., 2004) all of these models and suggestions are, for the most part, based on the interpretation of static histological data rather than direct lineage analysis. According to Dor et al., pre-existing beta cells, rather than pluripotent stem cells, are the major source of new beta cells during adult life and after pancreatectomy in mice.

1.2.2. Beta cell proliferation on demand. Factors, exerting stimulatory proliferative effect on beta cells.

In adulthood there is little increase in the β cell number in a population of normal adult pancreatic islet cells that are well differentiated - the number of beta cells undergoing cell division has been measured to be between 0,5 and 2 % (Swenne and Andersson 1984). The replication rate of beta cells appears to be determined by the number of cells able to enter into the cell division cycle (G1, S, G2 and M phases) from the quiescent G0 phase (Swenne 1992). Pancreatectomy, or removal of pancreas, is a very useful approach to demonstrate the regenerative potential of beta cells. 60% partial pancreatectomy does not result in glucose intolerance or permanent diabetes (described below). This maintenance of glucose homeostasis is paritally due to a regeneration amoung the remaining beta cells (Liu et al., 2000; Lohr et al., 1989). However, when 85-90% partial pancreatectomy is performed, mild hyperglycemia ensues which is followed by increased beta cell replication and a 40%

increased beta cell mass (Bonner-Weir S. et al., 1983). Interestingly, 95% pancreatectomy results in severe hyperglycemia with non-existent or very minor signs of beta cell replication (Clark et al., 1982). Based on the pancreatectomy models, it is evident that beta cells have a certain regenerative capacity.

One of the most convincing examples for the dynamic nature of beta cell mass is the adaptation of beta cells during pregnancy due to the increased demand for insulin. During rat pregnancy there is almost 50% increase in beta cell mass, with increased beta cell replication, larger islets and even hypertrophied beta cells (Marynissen et al., 1983). This is due to increased mitogenic activity of beta cells exposed to placental lactogen (PL), prolactin (PRL), and growth hormone (GH) (Svenstrup et al., 2002; Parsons et al., 1995; Parsons et al., 1992) that are considered as primary regulators of beta cell proliferation (Friedrichsen et al., 2001). With delivery, there are marked changes in the hormonal milieu and decreased demand for insulin. There is a marked involution over the period of lacation that returns beta cell mass to non-pregnant levels (Scaglia et al., 1995; Parsons et al., 1995).

Evidence for a mitogenic role of PL has been given in the rat where the circulating levels of the two PL's are maximal just prior to the maximal mitotic activity in the islet cells (Parsons et al., 1992). Sera from pregnant women stimulate DNA synthesis in cultured mouse islets (Nielsen et al., 1986) and amnionic fluid stimulates, DNA synthesis in fetal rat islets (Nielsen et al., 1986). Culture of rat islets in the presence of GH, PRL or PL was found to stimulate both DNA synthesis and insulin production (Nielsen et al., 1982). It has been shown that the effect of GH and PRL on beta cell replication is mediated through JAK/STAT pathway (Nielsen et al., 2001). GH-induced beta cell proliferation is glucose dependent (Cousin et al., 1999). Glucose has no independent effect on JAK2/STAT5 activation and it is yet unclear how glucose provides a means to permit GH action, although it has been suggested that Ca^{2+} is possible mediator (Sekine et al., 1998; Cousin et al., 1999). When rat islets were maintained in tissue culture at widely different glucose concentrations, it was found that the

cell cycle proceeded at the same rate irrespective of the ambient glucose concentration (Hellerström et al., 1985). This gave rise to the proposal that glucose enhances beta cell replication by expanding the pool of beta cells capable of entering the cell cycle.

Other factors, which lead to a stimulation of beta cell replication, are amino acids (Swenne et al., 1980), lithium (Sjoholm et al., 1992), the phorbol ester 12-O- tetradecanoylphorbol 13-acetate (TPA) (Sjoholm et al., 1991), nicotinamide (Sandler et al., 1986).

GLP-1 (glucagons-like peptide-1) is a hormone derived from post-translational processing of proglucagon, and is secreted from enteroendocrine alpha cells of the intestine. GLP-1 augments insulin secretion and lowers blood glucose to physiologic levels. In pancreatic beta cells and acinar cells, it binds to the GLP-1 receptor and increases intracellular cAMP, leading to activation of proteinkinase A (PKA). In the GLP-1 receptor knockout mice, islet abnormalities are observed and glucose tolerance is impaired (Flamez et al., 1999), suggesting that GLP-1 signaling plays a crucial role in the maintaince of normal islet function and glucose tolerance. The importance of GLP-1 for stimulation of islet cell proliferation was originally demonstrated in lean 20-day old normoglycemic mice (Edvell et al., 1999). Afterwards, several studies using *in vivo* models showed that GLP-1 could regulate islet growth mainly by controlling beta cell neogenesis (Tourrel et al., 2001).

Other studies demonstrated that treatment of diabetic Goto-Kakizaki rats with GLP-1 or extendin-4[4], resulted in stimulation of beta cell neogenesis and proliferation with persistent expansion of beta cells mass detected at adult age (Tourrel et al., 2002). Altogether these studies suggest that GLP-1 and its analogs exert a major trophic role on pancreatic beta cells and their use gives the possibility to pharmacologically stimulate beta cell neogenesis from precursor cells.

Discovering ways to stimulate beta cell proliferation and differentiation may lead to new ways of increasing the beta cell mass either in vivo in the diabetic patient or in vitro by propagation of cells for transplantation.

[4] Extendin-4 is an agonist of GLP-1 and similarly to GLP-1 has also very potent insulinotropic effects. In contrast to GLP-1, extendin-4 has a much longer in-vivo half-life, which makes it a good substance to examine the in-vivo effects of GLP-1 signalling (Letters to the editor, *Diabetes research and clinical practice*, 1999)

2. Diabetes Mellitus

Diabetes mellitus is one of the most common metabolic diseases. It is characterized by disturbances in carbohydrate, lipid, and protein metabolism, and it currently afflicts more than 200 million people worldwide.

Etiologically, diabetes can be classified as type 1 diabetes, type 2 diabetes, mature-onset diabetes in the young (MODY), gestational diabetes, and other types of diabetes that cannot be included into any of the previous categories.

2.1. Type 1 Diabetes Mellitus

Type 1 Diabetes Mellitus (also referred to as ' insulin-dependent diabetes mellitus (IDDM)', or juvenile-onset diabetes') is responsible for 5-10% of the total cases of diabetes. The disease may become manifested at any age, even though its incidence has a peak in early adolescence. It results from the autoimmune destruction of insulin secreting beta cells.

Autoantibodies against islet cell antigens, including GAD65, ICA512, insulin, and other proteins are found in most patients before the onset of the disease, and their titer most often decreases once beta cells have been destroyed.

Both genetic and environmental factors are implicated in the development of type 1 diabetes, but their precise role is not fully understood. Genetic risk factors for type 1 diabetes are variations in the human leukocyte antigen (HLA) genes that encode proteins responsible for antigen presentation to T-cells, and in the non-HLA genes that encode insulin and cytotoxic T-lymphocyte-associated antigen (CTLA4) (Steck et al., 2005).

2.2. Type 2 Diabetes Melitus

Type 2 Diabetes Mellitus (also referred to as 'non-insulin-dependent diabetes mellitus (NIDDM)', or 'adult-onset diabetes') is characterized by insulin resistance concomitantly with deficits of beta cell mass and insulin secretion relative to metabolic demands. The onset of type 2 diabetes is commonly preceded by reduced glucose tolerance, which does not however necessarily always progress to become clinically manifested. Glucose intolerance is a metabolic state which is intermediate between the normal and the diabetic condition, and is defined by fasting glucose levels ≥ 110mg/dl, but ≤126 mg/dl, above which diabetes is present. This type of diabetes has a strong genetic predisposition and its onset typically occurs later in life. Two suscepptibility genes have been identified so far, namely the protease calpain 10 and the transcription factor hepatocyte nuclear factor-4 (HNF4). The factors responsible for beta cell deficiency are still unknown. Type 2 diabetes is often associated with obesity and hypertension. The concomitant occurrence of these disorders is commonly referred to as 'metabolic syndrome'.

2.3. Mature-Onset Diabetes in the Young

Mature-Onset Diabetes in the Young (MODI) has similar clinical characteristics to type 2 diabetes. MODY is caused by a mutation of a single gene that is inherited as an autosomal trait. Mutations in the genes encoding hepatic nuclear factor 4 (HNF4), glucokinase (GCK), hepatic nuclear factor 1 alpha and beta (commonly known as HNF1A and HNF1B, or TCF1 and TCF2, respectively), insulin promoter factor-1 (IPF-1), and BETA2/NeuroD1 are the cause of the six known forms of MODY.

2.4. Gestational Diabetes Mellitus

During pregnancy, the maternal placenta supplies the growing fetus with nutrients and produces a variety of hormones. On one hand, some of these hormones such as steroids and the placental lactogen counteract the action of insulin. On the other hand, increased metabolic demand and placental lactogen itself promote an expansion of the beta cell mass(described above), hence allowing the secretion of more insulin. Gestational diabetes mellitus occurs when such a compensatory mechanism is defective, i.e. the mother's production and/or secretion of insulin are not sufficient to overcome the insulin resistance induced by the placental hormones.

2.5. Islet Transplantation – a possible treatment for diabetes

The usual objective of pancreas or islet transplantation[5] is to restore endogenous insulin secretion **t**o a diabetic individual by providing the functioning beta cells missing from the recipient's own pancreas. Exogenous insulin therapy has risks of insulin-induced hypoglycemia and hyperinsulinemia-induced atherogenesis and hypertension (Jun and Yoon, 2005).

Therefore precise physiological regulation of blood glucose levels is required to remove these complications.

[5]The first clinical attempt at islet transplantation occurred on 20 December 1893 in Bristol, UK, 28 years before the discovery of insulin. Dr.Watson-Williams and his surgical colleague, Mr.Harsant, transplanted three pieces of freshly slaughtered sheep's pancreas, 'each the size of a Brazil nut', into the subcutaneous tissues of a 15-year-old boy dying from uncontrolled ketoacidosis. The operation, performed under chloroform anaesthesia, was completed 'within twenty minutes of the death of the sheep'. Although there was a temporary improvement in glucose excretion before the boy's death 3 days later, this xenograft was destined to fail without immunosuppression. The idea was not new, for Oscar Minkowski had carried out a similar procedure in a pancreatectomized dog in 1892 and had described a temporary reduction in glycosuria (Shapiro J., 2001)

Transplantation of new insulin-producing beta cells, in the form of the whole pancreas or isolated islets, has been shown to ameliorate the disease by eliminating the need for exogenous insulin and normalizing glycosylated hemoglobin levels (Kaufman and Lowe, 2003). Islet transplants are a particularly attractive form of therapy because they are a minimally invasive procedure and are more easily to be scaled-up to treat the large numbers of people affected by diabetes (Sutherland et al., 2004). Currently, only a handful of programs have been successful in the endeavor. Nevertheless, the early clinical experience strongly demonstrates that islet transplantation is an effective treatment strategy in selected patients with type 1 diabetes.

3. Mutual signaling or intercellular crosstalk between tissues and endothelial cells

The endothelium of blood vessels has traditionally been regarded as rather inert 'plumbing', which enables metabolic exchange between blood and tissues by virtue of its permeability and proximity to all cells. In recent decades, however, there has been a reassessment of the role of the endothelium, suggesting that it is "more than a sheet of nucleated cellophane" (Florey, 1966). Endothelial signals are now implicated in the regulation of many processes, and endothelial cells (ECs) are known to communicate directly with adjacent cells (Figure 2).

Different studies have shown that the endothelium provides developmental signals to organs and differentiating cells (Majumdar and Drummond, 1999; Lammert et al., 2001; Matsumoto et al., 2001; Lammert et al., 2003; Yoshitomi et al., 2003 and Figure2) Although it is well known that tissues signal to ECs, providing cues for the patterning of vessels (Stewart et al., 1981, Aird et al., 1997), some studies have identified reciprocal signals from ECs back to surrounding tissues (Kitamoto et al., 1997; Gerber at al., 1999).

The possibility of EC signaling is not entirely surprising, given that ECs express numerous secreted and cell-surface signaling molecules and are intimately associated with developing tissues, growing and changing with them in a coordinated manner (Cleaver and Melton, 2003). Immediate physical contact between endothelial and tissue cells occurs both in the early embryo, and later in capillary beds in the adult. It is therefore possible that mutual signaling between endothelium and tissues during development forms the molecular basis for interdependent physical and physiological relationships that last into adulthood.

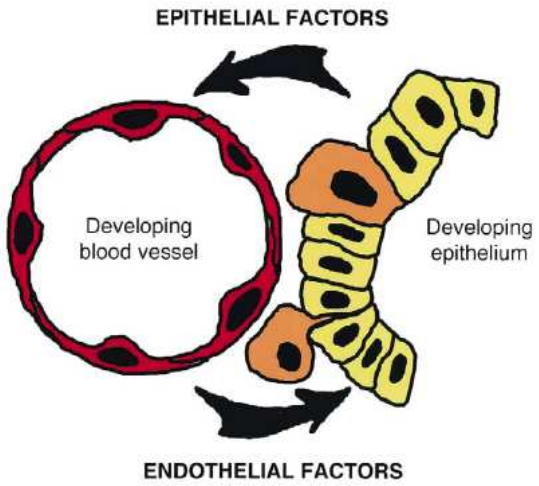

Figure2. Endothelial-epithelial signaling. Epithelial cells and Endothelial cells might codevelop as a result of mutual signaling event between these cell types. ECs differentiate, proliferate and form blood vessels as a response to epithelial factors. In turn, epithelial cells respond to endothelial signals with growth, differentiation and delamination (Lammert et al., 2003).

3.1. Mutual signaling between pancreatic beta cells and endothelial cells – observations in the mouse embryo and in adult pancreatic islets

Several studies demonstrated the importance of endothelial signals during pancreatic development (Lammert et al., 2001; Yoshitomi and Zaret, 2004). Lammert and colleagues examined how endothelium influences the development of the pancreas in both the mouse and the frog Xenopus. In vivo embryonic manipulation of frog embryos to block the formation of the dorsal aorta endothelium leads to failure of pancreatic gene expression in the underlying endoderm. This indicated that EC signals are required for pancreatic differentiation. Conversely, using transgenic mice expressing VEGF-A under the control of the PDX1 promoter, EC signals were shown to instruct cells in the foregut to differentiate into pancreatic beta cells. Excess ECs were attracted to stomach and duodenal epithelium early during mouse development. Ectopic insulin expressing cells, as well as bud-like structures were observed at locations where the epithelium of stomach and duodenum contacted ectopic ECs. This shows that during early embryonic development, ECs can instruct PDX1-expressing foregut epithelium to adapt a pancreatic cell fate. In vitro recombination experiments were also conducted to exclude the possible effects of altered nutrition and gas exchange. Dorsal endoderm and aortic endothelium from early embryos were cultured together, resulting in pancreatic induction as detected by insulin gene expression. In contrast, recombination of the same endoderm with other tissues did not. These coculture experiments showed that endothelial signals were sufficient for inducing the pancreatic program, at least in this assay with a relatively prepatterned endoderm. By using *Flk1*$^{-/-}$ mouse embryos and tissue recombination experiments Yoshitomi and Zaret discovered that dorsal pancreatic bud emergence and the maintenance of Pdx1 expression require aorta or endothelial cell interactions. Yoshitomi and Zaret also found that endothelial cells are necessary for the induction of insulin and glucagon genes. All these studies showed the influence and importance of endothelial signals for induction of endocrine cell differentiation

and morphogenesis. On the other hand, other studies, by deleting VEGF-A in the mouse pancreas showed that endocrine cells signal back to the adjacent endothelial cells to induce the formation of a dense capillary network in islets (Lammert et al., 2003). Based on their studies Lammert et al., proposed a two-step model in which the sequential and reciprocal signaling between endothelial cells and endocrine cells leads to the formation of functional pancreatic islets.

4. Vascular niches

The embryonic EC-tissue interactions lead to the question of whether these interactions also play a role in the adult. It is known, for example, that during regeneration both vascular and nonvascular cells develop and differentiate. Whether similar or different inductive EC signals play a role during tissue regeneration is unclear at the moment. However, reports on the coordinated interaction of neurons and blood vessels in the brain need to be mentioned as an example of endothelial signals during adult tissue remodeling and regeneration (Louissaint et al. 2002; Palmer et al. 2000).

In mammals, the hippocampus is responsible for learning and memory, and neurons residing in the hippocampus therefore have to be continuously remodeled (in part by cell death and growth). Adult neurogenesis is mediated by stem cell-like precursors that remain in the hippocampus. Neural and endothelial precursors proliferate together in the subgranule zone, an area known to generate new neurons throughout adult life. Tight clusters of these cells, consisting of proliferative aggregates of neural precursors, committed neuroblasts, glia and angioblasts, are commonly found at branch points or termini of fine capillaries. Precursors in other areas of the hippocampus are also proliferative, but they are not found in immediate juxtaposition to vascular endothelium and do not generate neurons. This has led to the

hypothesis that the angiogenic microenvironment (or "**vascular niche**") might be important for adult hippocampal neurogenesis and ultimately for hippocampus-mediated cognitive function (Palmer et al. 2000). This study is the first evidence that adult neurogenesis occurs within an angiogenic niche.

The neurogenic effect of endothelial cells could not be mimicked by fibroblasts or by vascular smooth muscle cells (Shen et al., 2004), indicating that not all cell types alter the neural stem cell differentiation profile. There is evidence that endothelial cells enhance neurogenesis, possibly through the secretion of brain-derived neutrophic factor (Louissaint et al., 2002; Mi et al., 2001). But endothelial cells are not limited to instructing the neural lineages – they also promote formation of pancreatic and liver tissue independently of their ability to form vasculature (Lammert et al., 2001; Matsumoto et al., 2001). The last two studies imply that differentiation of stem cells into hepatocytes or pancreatic islet cells might require the contribution of inducing factors secreted by endothelium. Endothelial signals have also been identified during the development of various other tissues, such as adipose and bone tissue. In the embryo, an established vascular network precedes the formation of adipose tissue and both tissues grow coordinately throughout life. In vitro experiments have shown that both soluble and insoluble vascular extracellular matrix can drive the proliferation and differentiation of adipocyte precursors (Varzaneh et al., 1994; Hutley et al., 2001). Defects in endothelial – myocardial communication are believed to underlie a variety of human congenital cardiac disorders such as transposition of the great arteries, atrioventricular septal defects (Nakajima et al., 2000). Altogether, these studies suggest that EC-tissue interactions play an important role in tissue and organ formation as well as tissue regeneration in the adult.

Understanding the signaling events within the 'vascular niches' could be of widespread therapeutic importance. It was recently shown that the dense vascular network in the islets is required for proper endocrine function and islet size (Lammert et al., 2003). During diabetes

mellitus pancreatic islet function is disturbed, and various attempts have been made to either stimulate islet function or regrow islets for the tretment of diabetes. It is also known that islet microvasculature is destroyed during islet islolation procedure. Transplanted pancreatic islets, unlike transplanted solid organs, must revascularize to survive and function (Jansson and Carlsson, 2002), and this process of angiogenesis is likely an important factor that ultimately determines whether an islet transplant secretes sufficient insulin to reverse diabetes. Because of the importance of endothelial signals during pancreatic development and the necessity of proper vascularization in adult islets it is very likely that pancreatic blood vessel endothelium has some other roles besides enabling the metabolic exchange between the blood and islet tissue. Given the importance of endothelial signals for adult neurogenesis and also the neuroendocrine origin of the beta cells it is tempting to speculate that similar signals could be important for maintaining proper adult beta cell mass and function.

5. Basement membrane

Tissues are not made up solely of cells. A substantial part of their volume is extracellular space, which is largely filled by an intricate network of macromolecules constituting the extracellular matrix (Alberts, Molecular biology of the cell, 2002). A particular extracellular structure refered to as basement membrane or basal lamina is found in all animal phyla and is already produced by early stages of embryonic development. Transmission electron microscopy typically reveals a sheet-like structure of 50-100 nm thickness, with an amorphous appearance (Timple and Brown, 1996). Such morphological structures are usually found underlying sheets of epithelial and endothelial cells and surrounding most muscle cells, fat cells and peripheral nerve axons (Vracko, R., 1974). Originally believed to serve as a selective barrier and scaffold to which cells adhere, it has become evident that the individual

components of the BM are regulators of biological activities such as cell growth, differentiation, and migration, and that they influence tissue development and repair (Aumailley and Krieg 1996; Timpl 1996; Aumailley and Gayraud 1998). Although BMs are widespread tissue components, their fine structure and composition varies from tissue to tissue, as well as within the same tissue at different developmental periods and during repair. All BMs contain Type IV collagen, laminins, entactin-1/nidogen-1, and heparan sulfate proteoglycans. Collagen IV and laminin are the major constituents of all basement membranes. Both proteins exist as multiple isoforms and each form a huge irregular network by self assembly. These networks are connected by nidogen, which also binds to several other components (proteoglycans, fibulins) (figure 3).

What is now known as laminin 1 was first discovered over 20 years ago in the matrix formed in a murine sarcoma (the mouse Engelbreth-Holm-Swarm sarcoma). The molecule appears to be between 200 and 400 kDa (Bosman and Stamenkovic, 2003), is composed of three disulphide linked chains, and has a characteristic cross shape. Molecular cloning of the three chains (now known as α1, β1, and γ1) of laminin 1 (LN-111) (Aumailley et al., 2005) led to the discovery of a variety of homologues. As yet, five α chains, three β chains and three γ chains have been identified (Aumailley et al., 1998; Colognato H et al., 2000; Miner et al., 1997).

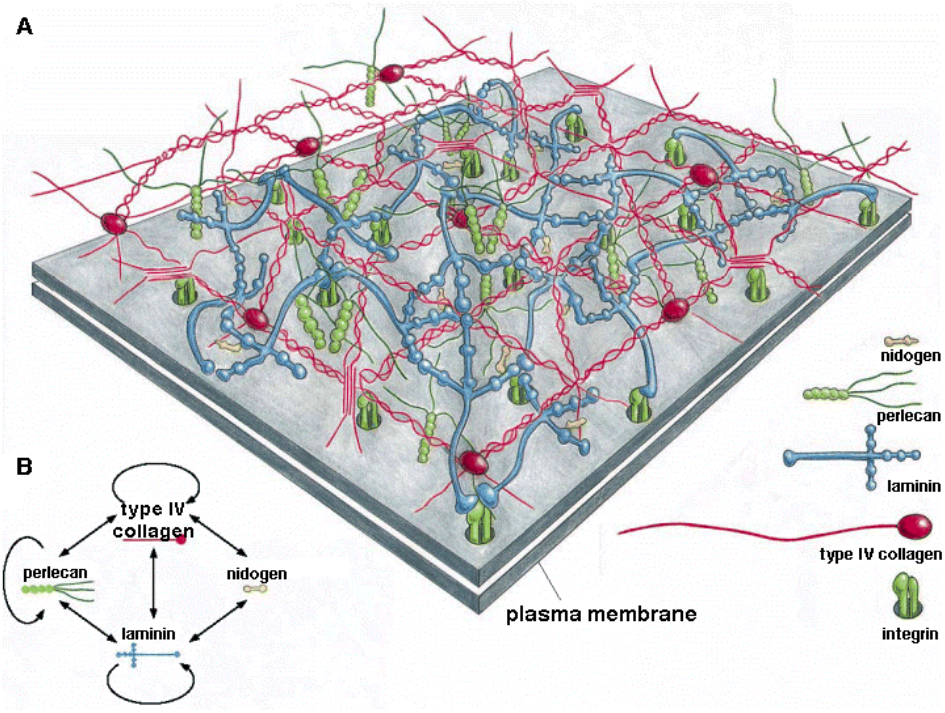

Figure 3. A model of the molecular structure of a basal lamina. (A) The basal lamina is formed by specific interactions between (B) the proteins type IVcollagen, laminin, and nidogen, and the proteoglycan perlecan. Arrows in (B) connect molecules that can bind directly to each other. There are various isoforms of type IV collagen and laminin, each with a distinctive tissue distribution. Transmembrane laminin receptors (integrins and dystroglycan) in the plasma membrane are thought to organize the assembly of the basal lamina; only the integrins are shown. (Based on H. Colognato and P.D. Yurchenco, Dev. Dynamics 218:213–234, 2000.) (from Molecular Biology of the Cell, Alberts et al., 2002)

The increase in complexity of the laminin family in mammals with differential temporal/spatial expression patterns poses interesting questions about the requirements for the development of functional diversity in the family. Currently, 16 trimers are known from mouse and human, if the major splice variants and a partially described variant are included (Aumailley et al., 2005). Laminin nomenclature is described in the chapter ″Materials and Methods″ of this work.

Laminin isoforms are synthesized by a wide variety of cells in a tissue-specific manner. Like type IV collagen, laminins can self-assemble in vitro into a feltlike sheet, largely through

interactions between the ends of the laminin arms. As nidogen and perlecan can bind to both laminin and type IV collagen, it is thought that they connect the type IV collagen and laminin networks (Figure 3). In tissues, laminins and type IV collagen preferentially polymerize while bound to receptors on the surface of the cells producing the proteins. Many of the cell-surface receptors for type IV collagen and laminin are members of the integrin family (described below). Another important type of laminin receptor is the transmembrane protein dystroglycan, which, together with integrins, may organize the assembly of the basal lamina. Notably, virtually all epithelial cells synthesize laminin, as do smooth, skeletal, and cardiac muscle, nerves, endothelial cells, bone marrow cells, and the neuroretina (Bosman and Stamenkovic, 2003). Laminins appear to modulate the behaviour of adjacent cells, including cell adhesion, cell migration, and cell differentiation.

Collagen type IV of the chain composition $\alpha1(IV)_2\alpha2(IV)$ (Pöschl et al., 2004) is a widespread basement component and is considered to be responsible for the mechanical stability of basement membrane (Hudson et al., 1993). An important characteristic of the collagen IV sequence is the presence of several single cycteins, which are used for intra- and intermolecular disulfide bridges.

Basement membrane proteoglycans (PG) were originally found in the kidney glomerulus (Kanwar and Farquhar 1979). PGs in the basement membrane putatively play a structural role in maintaining tissue histoarchitecture (Reinhardt et al. 1993; Costell et al. 1999; Hopf et al. 1999), aid in selective filtration processes (Miettinen et al. 1986), sequester growth factors (Aviezer et al. 1994) and extracellular ions (Lerner and Torchia 1986), and help regulate cell differentiation (Li et al. 1987). Some of these roles have been shown for perlecan, but not much is known about the functions of other BM proteoglycans. Perlecan was the only large BM heparan sulfate proteoglycan described for many years. Perlecan null mice show no evidence of abnormalities until embryonic Day 10 (E10), and between E10 and 12 most embryos die with evidence of bleeding into the pericardial sac.

Basement membranes first appear in the preimplantation period of development in the mouse and during gastrulation in the nematode and fly. Members of the laminin family are required for basement membrane assembly, a process thought to depend upon both the shared and distinct architecture-building and cell-anchoring properties of these heterotrimers (Yurchenco P et al., 2002). Several studies now provide evidence that laminin forms the initial cell-anchored polymer required for basement membrane assembly and triggers processes required for epithelial cell polarization (Huang et al. 2003). As the laminin polymer accumulates on the target cell surface through a process of polymerization and anchorage, type IV collagen, nidogen, and perlecan are integrated into the structure. Laminin binds to nidogen-1, produced by the ectoderm, through a noncovalent high-affinity bond (Timpl and Brown, 1996). Nidogen, in turn, can bind to type IV collagen and other basement membrane components, forming bridging links. Type IV collagen forms a three-dimensional polymer that provides a second stabilizing basement membrane network (Yurchenco, 1994). Perlecan, a heparan sulfate proteoglycan, binds to nidogen-1 through its core protein and may bind to the G domain of laminin (Timpl and Brown, 1996). When laminin-1 is not secreted, the other basement membrane components accumulate in disorganized manner (Smyth et al. 1999 and Li et al. 2002).

Basement membranes have a great influence on tissue compartmentalization and cellular phenotypes from early embryonic development onwards. A critical function of basement membranes is to mediate cell adhesions to different cell types, a process related to cell polarization that contributes to cell organization and stability within developing tissues. In fact, the inability of cells to polarize and correctly associate with their neighbors in the nematode embryo likely accounts for the embryonic lethality in laminin-null animals (Huang et al., 2003). Moreover, in nematode and fly laminin mutants, tissues adhere to each other when they should not or fail to adhere when they should (Martin et al. 1999; Huang et al. 2003). The mouse laminin-α5 knockout results in late embryonic lethality with neural tube,

limb, placental vasculature, and kidney defects (Miner et al., 1998). Further analysis of the kidney phenotype has revealed that during the capillary loop stage when a switch occurs from α1 to α5 expression in the glomerular basement membrane (GBM), the endothelial cells and mesangium are extruded and the glomerulus fails to develop (Miner and Li, 2000). Related to these polarity and adhesion defects is the ability of some cells to invade neighboring tissues, disrupting tissue organization and integrity.

Originally believed to serve as a selective barrier and scaffold to which cells adhere, it has become evident that the individual components of the BM are regulators of biological activities such as cell growth, differentiation, and migration, and that they influence tissue development and repair (Aumailley and Krieg 1996; Timpl 1996; Aumailley and Gayraud 1998; Schwarzbauer, 1999).

Collectively, many studies argue for a pivotal role of basement membrane laminin in early embryogenesis and adult life subsequently. How exactly basement membrane components are involved in different processes still remains to be understood at the molecular level.

Integrins

Integrins are the major metazoan receptors for cell adhesion to extracellular matrix proteins, and in vertebrates, also play important roles in certain cell-cell adhesions. In addition to mediating cell adhesion, integrins make transmembrane connections to the cytoskeleton and activate many intracellular signaling pathways. Since the recognition of the integrin receptor family around 20 years ago (Hynes, 1987), they have become the best-understood cell adhesion receptors (Hynes, 2002). Integrins and their ligands play important roles in development, cell division, survival, apoptosis, immune responses, leukocyte traffic, and cancer, and are at the heart of many human diseases—genetic, autoimmune, and others.

Integrins are a family of heterodimeric transmembrane receptors consisting of two noncovalently associated transmembrane glycoprotein subunits called α(120-180 kDa) and β(90-110 kDa) (Figure 4). The binding of integrins to their ligands depends on extracellular divalent cations (Ca2+ or Mg2+, depending on the integrin), reflecting the presence of divalent-cation-binding domains in the extracellular part of the α and β subunits.

The type of divalent cation can influence both the affinity and the specificity of the binding of an integrin to its ligands.

18 different α and 8 different β subunits are known in mammals, which can combine to 24 different integrin receprtors (Brakebusch et al., 2002; Hynes RO, 2002), (Figure 5). Most integrins recognize several ECM proteins. Conversely, individual matrix proteins, such as laminins, collagens and fibronectin bind to several integrins (Hynes RO, 1987; Rouslahti and Pierschbacher, 1987), (Figure 5).

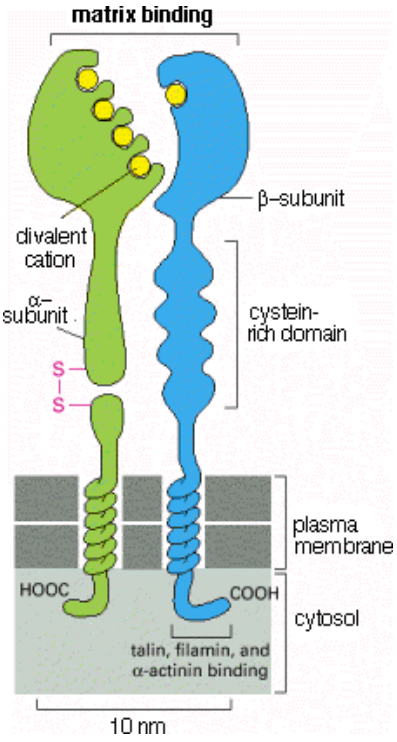

Figure 4. The subunit structure of an integrin cell-surface matrix receptor. Electron micrographs of isolated receptors suggest that the molecule has approximately the shape shown here, with the globular head projecting more than 20 nm from the lipid bilayer. Each integrin molecule binds to a matrix protein outside the cell and to the actin cytoskeleton (via the anchor proteins indicated) inside the cell. The α and β subunits are held together by noncovalent bonds. The extracellular part of the β subunit contains a single divalent-cation-binding site, as well as a repeating cysteine-rich region, where intrachain disulfide bonding occurs. The integrin extracellular domain binds to components of the extracellular matrix, while the cytoplasmic tail of the β subunit binds indirectly to actin filaments via several intracellular anchor proteins (adapted from from Molecular Biology of the Cell, Alberts et al., 2002)

Each αβ combination has its own binding specificity and signaling properties. The largest subgroup is formed by the β1 subunit containing integrins which consist of 12 members with different ligand binding properties (Figure 5). They are found on almost all vertebrate cells: α5β1, for example, is a fibronectin receptor and α6β1 a laminin receptor. Mutant mice that cannot make any β1 integrins die at implantation, whereas mice that are only unable to make

the α7 subunit (the partner for β1 in muscle) survive but develop muscular dystrophy (as do mice that cannot make the laminin ligand for the α7β1 integrin).

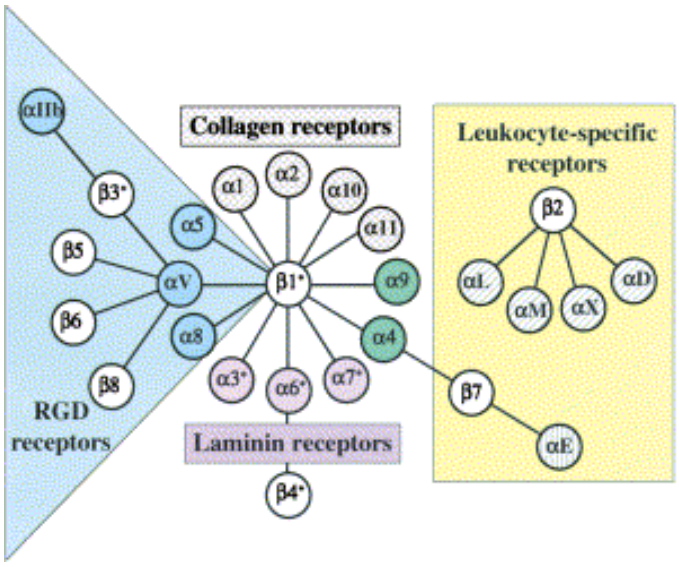

Figure 5. The Integrin Receptor Family. The figure depicts the mammalian integrin subunits and their αβ associations; 8 β subunits can assort with 18 α subunits to form 24 distinct integrins. These can be considered in several subfamilies based on evolutionary relationships (coloring of α subunits), ligand specificity and, in the case of β2 and β7 integrins, restricted expression on white blood cells. Fibronectin binding integrins are depicted as RGD receptors since cell surface binding domain of fibronectin molecule contains the tripeptide Arg-Gly-Asp (RGD). (adapted from Hynes RO., *Cell*, 2002

Besides their role in providing physical support for the cells, integrins transduce signals through the cell membrane in two opposite directions. The binding of integrins to the extracellular matrix proteins elicits signals that are transmited into the cell (outside-in-signaling), while signals generated inside the cell can regulate the binding activity of the integrins to their ligands outside the cell (inside-out signaling) (Giancotti and Ruoslahti, 1999).

Integrin-ligand interactions trigger a spectrum of signal transduction pathways with profound effects on cell survival, cell proliferation and gene transcription. As integrins have very short cytoplasmic tails without enzymatic activity, they connect to these pathways by affecting other signaling and adaptor proteins.

As integrins bind to ECM, they become clustered in the plane of the cell membrane and associate with cytoskeletal and signaling complexes (Giancotti and Ruoslahti, 1999). This event triggers a series of biochemical signals, such as activation of focal adhesion kinase (FAK) and its downstream effectors, activation of the Ras-extracellular signal-related kinase (ERK) cascade, and activation of phosphatidylinositol 3-kinse (PI3-K) and proteins of the Rho family (Fig. 6). Integrin-dependent autophosphorylation of FAK allows it to interact with docking or adaptor proteins, including paxilin, tensin and Grb2/Son of Sevenless ('SOS'), which in turn are able to activate downstream signaling mediators previously implicated in growth control, including, Src, Ras, and Raf (Juliano R., 1996). FAK also can activate activate signaling from PI3K to Akt/protein kinase B (PKB) through phosphatidylinositol-3,4,5-triphosphate (Giancotti and Ruoslahti, 1999). Mutations of tyrosine residues critical for FAK autophosphorylation prevents integrin-mediated proliferation (Schlaepfer and al., 1994).

Integrin-induced signaling can overlap or even synergize with those of growth factors (EGF, PDGF, FGF) and cytokines. There are studies, which identify different mechanisms for the regulation of growth factor receptor regulation by integrins, indicating multiple ways of achieving cooperation between these major signaling systems. One general mechanism involves a collaborative interaction between growth factor and integrin ligands (Schwartz and Baron, 1999; Yamada and Danen, 2000-Humana press). An alternative viewpoint proposes that the direct activation of growth factor receptors by integrins can occur in the complete absence of any growth factor ligand and this activation seems to be FAK-independent (Moro et al., 2002; Wang et al., 2001).

In addition to growth factor receptor activation, adhesion of cells to different basement membrane proteins, including collagen, laminin-5, fibronectin, tenascin, also leads to activation of mitogen-activated protein kinase (MAPK) pathway (Schlaepfer et al., 1994; Chen et al., 1996; Yokosaki et al., 1996). Activation of the MAPK signal transduction pathway provides a common route leading to transcriptional regulation of genes that are critical for cell growth and differentiation. Family members that are sequentially activated following transient activation of Ras GTP-binding proteins via receptor-tyrosine kinases include MAPK/ERK kinase (MEK and ERK1 (p44)/ERK2 (p42) (Hill and Treisman, 1995). Subsequently it was shown that MEK-dependent phosphorylation of ERK1 and ERK2 results in their translocation to the nucleus, where they phosphorylate and activate a number of transcription factors associated with early-response genes (Hill and Treisman, 1995; Segar and Krebs, 1995).

Other studies, however, suggest that integrin activation of MAPK may be independent of FAK or Ras, therefore implying the use of alternative or parallel pathays (Chen et al., 1996; Lin et al., 1997.To date, however, little is known about how or when cells may use these alternative routes.

An important mechanism by which cells regulate integrin function is through tight spatial and temporal control of integrin affinity for extracellular ligands. This is achieved by rapid, reversible changes in the conformation of the extracellular domains of the integrin heterodimer, so called integrin activation (Sims et al., 1991; Woodside et al., 2001). The integrin activation or the control of integrin affinity for ligands is usually mediated through the integrin β subunit cytoplasmic tail and can be regulated by many different signaling pathways (Tadokoro et al., 2003).

Intracellular signals switch integrins into a ligand-competent state as a result of elicited conformational changes in the integrin ectodomain (Xiong et al., 2003). Despite significant recent advances, the exact nature of the conformational changes leading to integrin activation

remains controversial (reviewed by Hynes, 2002; Liddington and Ginsberg, 2002; Lidington (2002); Xiong et al., 2003; Humphries et al., 2003). Changes in integrin avidity (clustering) complement the changes in affinity and appear to be critical for initiating integrin induced signaling (Hato et al., 1998). Changes in integrin affinity are not necessarily accomplished by changes in avidity and vice versa (Sebzda et al., 2002; Piccardoni et al., 2004).

Cellular control of integrin activation plays important roles in health and disease throughout development and during the course of adult life.

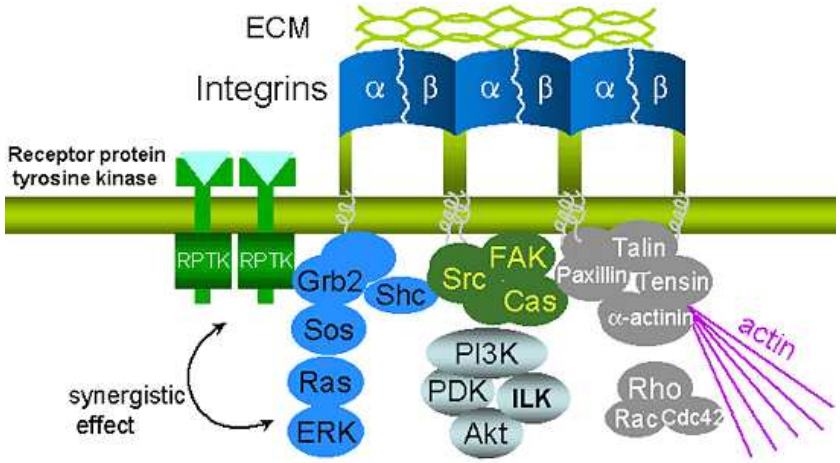

Figure 6. The association of integrin and ECM can activate several signaling pathways. Integrins signal predominantly through the recruitment and activation of Src-family kinases (Src). Most integrins recruit focal adhesion kinase (FAK). Very often the signals are transduced toward phosphatidylinositol 3-kinase (PI3K) and subsequently to Akt/protein kinase B. Src phosphorylates Cas and paxilin, which results in the activation of Rac – an important cell cycle regulator. FAK can activate ERK/MAPK pathway by recruiting the growth-factor-receptor-bound-2 (GRB2) and son-of-sevenless (SOS) complex. Cooperative integrin-receptor tyrosine kinase (RPTK) signaling is required for regulation of cell proliferation (adapted form 'Beyond glycogenes', Gu J; Guo and Giancotti, 2004).

AIM OF THE THESIS

Several studies demonstrated the importance of endothelial signals during pancreatic development (Lammert et al., 2001, Yoshitomi and Zaret, 2004). The importance of embryonic beta cell-endothelial cell interactions raises the question of whether similar interactions also play a role in the adult. It was recently shown that the dense vascular network in the islets is required for proper endocrine function and islet size (Lammert et al., 2003). Identification of the nature of endothelial signals and the mechanism by which they regulate β-cell function and proliferation is the central topic of this thesis.

Since it was demonstrated that glucose stimulated insulin secretion *in-vitro* is improved when primary beta cells were cultured on laminin-5 rich matrix (Bosco et al., 2000), during my PhD thesis work I considered the vascular basement membrane as a possible sourse of endothelial signals that regulate β-cell function. My work identifies the protein components of the vascular basement membrane, and characterizes the way that they influence β-cell function, in particular insulin gene transcription. Because the β-cell mass correlates with the vascular density of the islets (Duvillie et al., 2002, Lammert et al., 2003) it is important to investigate if and how vascular basement membrane proteins regulate β-cell proliferation. All these questions are addressed by using islets from mice with pancreas specific VEGF-A deletion (Lammert et al., 2003) and also mouse insulinoma cell line (MIN6) (Myazaki et al., 1990) as model systems.

Understanding the mechanisms by which β-cells interact with endothelial cells might have an important impact on discovering ways to stimulate beta cell function and proliferation, which are impaired during diabetes

RESULTS

7. Basement membrane formation within pancreatic islets requires vascular endothelial cells

In an attempt to identify endothelial signals involved in endocrine function and islet growth, the vascular basement membrane was considered as a possible source of such signals. Mouse pancreas sections were stained with antisera raised against laminin and collagen IV, the major protein components of all basement membranes (Hallmann et al., 2005). Figure 7 shows that the basement membrane within islets is found exclusively around capillaries, but not islet cells, whereas exocrine acinar cells are surrounded by a continuous basement membrane (Figures 7A and 7C).

Previous differential expression analyses have shown that endocrine pancreatic precursor cells and islets down-regulate gene expression of many basement membrane proteins (Gu et al., 2004). To investigate whether beta cells require endothelial cells for synthesizing the intra-islet basement membrane, mice with a VEGF-A deletion[6] in pancreatic epithelial cells (Pdx1-Cre x VEGFloxP) were used (Lammert et al., 2003) (Figures 7B and 7D). The islets of these mutant mice harbor few or no endothelial cells compared with the islets of their heterozygous (control) littermates (Lammert et al., 2003). The loss of endothelial cells resulted in a loss of the intra-islet basement membrane (compare Figures 7B with 7A and 7D with 7C).

[6] Floxed VEGF-A can be specifically deleted in in the pancreas by expressing Cre recombinase under the control of the Pdx1 promoter (Offield et al., 1996), which is active in all pancreatic epithelial cells, but inactive in endothelial cells and mesenchymal cells (Gu et al., 2003).

However, the reduced number of endothelial cells affected neither the basement membrane of the exocrine acini nor the matrix of the islet capsule (Figures 7B and 7D). Thus, in contrast to acinar cells, beta cells are unable to form a basement membrane on their own and therefore require endothelial cells.

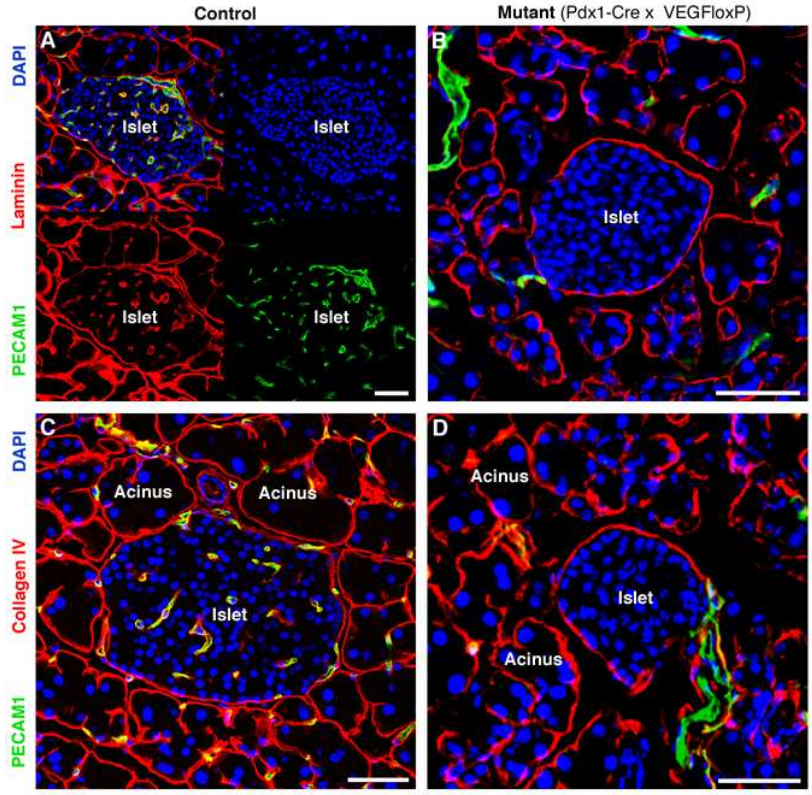

Figure 7. Basement membrane formation within pancreatic islets requires vascular endothelial cells. (A-D) Confocal images of mouse pancreatic sections show an islet surrounded by exocrine (acinar) pancreatic tissue. Endothelial cells were stained for the platelet-endothelial cell adhesion molecule PECAM1 in green (A-D), cell nuclei (DAPI) in blue (A-D), and the basement membrane proteins laminin (A, B) and collagen IV (C, D) in red. Some mutant islets (as shown in B, D) had neither endothelial cells nor basement membrane proteins, in contrast to control littermates (A, C). Scale bars, 50 μm. Note that mutant islets are smaller and shown at a higher magnification.

The observation that endothelial cells were required for basement membrane formation suggested that they also produced the major components of the basement membrane within

islets. Since laminin-411 (α4β1γ1) and laminin-511 (α5β1γ1) were described as the major laminin isoforms in vascular basement membranes (Sixt et al., 2001), mouse pancreas sections were stained for the laminin α4- and α5-chains. As shown in Figures 8A and 8B, both α-chains were strongly expressed around islet capillaries in contrast to laminin α1, which was not detectable in the vascular basement membrane (data not shown).

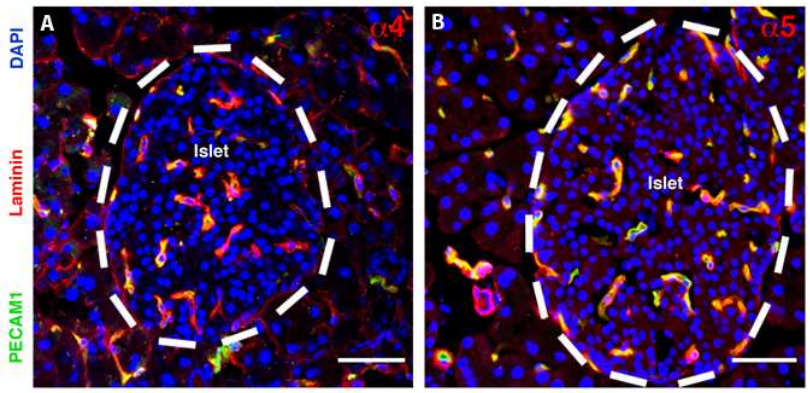

Figure 8. Immunohystochemical localization of the vascular-specific laminin chains - α4 and α5 within mouse pancreas. (A, B) Confocal images of mouse pancreas sections show wild-type islets (dotted lines) with surrounding exocrine tissue stained with DAPI (blue) and antibodies against PECAM1 (green) and laminin chains α4 (A), and α5 (B) (red). Scale bars, 50 μm.p

Next I looked at the gene expression of these laminin chains in endothelial cells and beta cells that were sorted from pancreatic islets by FACS (Figure 9). RNA expression analyses of the platelet endothelial cell adhesion molecule (PECAM1) as a marker for endothelial cells, and insulin-1 (Ins1) as a marker for beta cells, revealed no major cross-contamination between the sorted cell populations (Figure 9). Importantly, beta cells did not express any laminin α-chains, while endothelial cells expressed laminin chains α4 and α5 (Figure 9). Because either α1 or α5 is required for laminin secretion and basement membrane formation (Miner et al., 2004; Yurchenco et al., 1997; Yurchenco and Wadsworth, 2004), the conclusion is that beta cells cannot form a basement membrane. Additionally, islet endothelial cells, but not beta cells expressed collagen IV chains α1 and α2 (Figure 9). These

results demonstrate that beta cells use VEGF-A to attract endothelial cells, so that these cells can form a basement membrane adjacent to the beta cells.

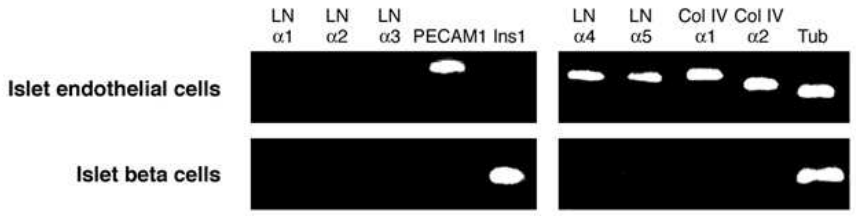

Figure 9. **Reverse transcription – polymerase chain reaction with mRNA isolated from mouse islet endothelial cells and beta cells.** The PCR reaction was performed with primers for all laminin (LN) α-chains, PECAM1, insulin 1 (Ins1), collagen IV (Col IV) α1- and α2-chains as well as α-tubulin (Tub) as a control.

8. Signals from the vascular basement membrane regulate insulin gene expression

Previous experiments showed that endothelial signals initiate insulin expression in the embryonic pancreas (Lammert et al., 2001; Yoshitomi and Zaret, 2004), thereby raising the question of whether endothelial signals also influence insulin expression in adult islets. As shown in figures 10B – 11A, beta cells in VEGF-A deficient mouse islets had less insulin secretory granules and reduced insulin gene expression levels in comparison with fully vascularized control islets (Figures 10A – 11A). In contrast, numbers of other organelles (Figure 10C), as well as expression of control genes (Figures11B and 11C), were not significantly changed.

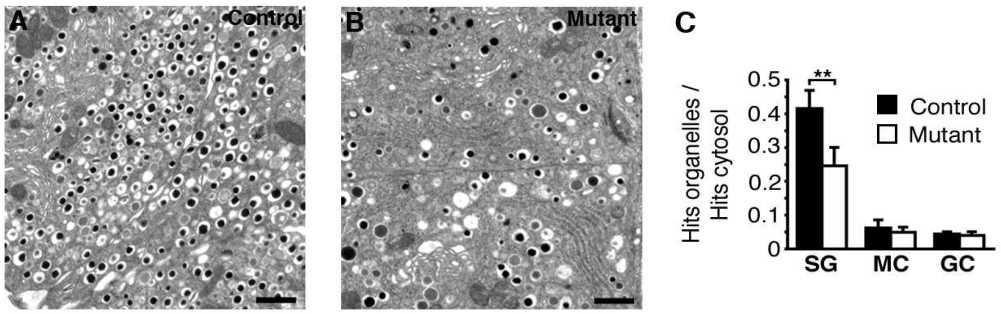

Figure 10. The lack of endothelial cells within pancreatic islets affects the number of insulin secretory granules. (A, B) Electron microscopic images of control islets (A) and mutant islets (B). Scale bars, 2 μm. (C) Quantification of the organelles in beta cells in relation to the cytosol in mutant mice (white bars) and control mice (black bars). SG, insulin secretory granules; MC, mitochondria; GC, Golgi complexes. 6-12 electron microscopic images of five control and five mutant islets were analyzed. **p < 0.005 (Student's t-test). All values are means ± SD.

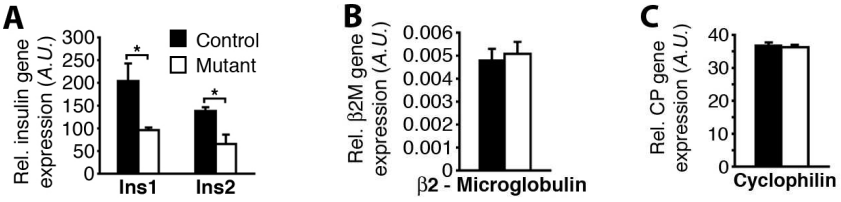

Figure 11. Reduction in insulin gene expression levels in VEGF-A deficient mouse islets. (A-C) Real-time RT-PCR analyses of both insulin genes (Ins1, Ins2) as well as control genes β2-microglobulin (β2M) and cyclophilin (CP) in mutant islets (white bars) and control islets (black bars) (n = 2 islet preparations). *p < 0.05, (Student's t-test). All values are means ± SD.

In vitro experiments were then performed to test if the vascular basement membrane contained signals capable of regulating insulin gene expression in beta cells (Figures 12 – 13). For these experiments mouse insulinoma cells (MIN6) were used. There was an increase in insulin gene expression levels when cells were plated on a reconstituted basement membrane, growth factor deprived matrigel (compare MG with UT in Figure12A). Since insulin gene expression did not change in response to a matrix formed by the interstitial

collagen I (compare Col I with UT in Figure 12A), the basement membrane must contain specific signals to enhance insulin gene expression. In an attempt to identify these signals, MIN6 cells were plated on surfaces coated with single basement membrane proteins (Figure 12B-12C). Among the proteins tested, laminin-111 (also called EHS-laminin or laminin-1; Table1 in 'Materials and Methods') up-regulated insulin gene expression most strongly (Figure 12B-12C). However, when this laminin was provided soluble to the media of the cells, higher concentrations had to be used, and insulin gene expression was increased to a lesser extent (Figure 12D), suggesting that beta cells needed to interact with a critical density of laminin molecules in order to activate insulin gene expression.

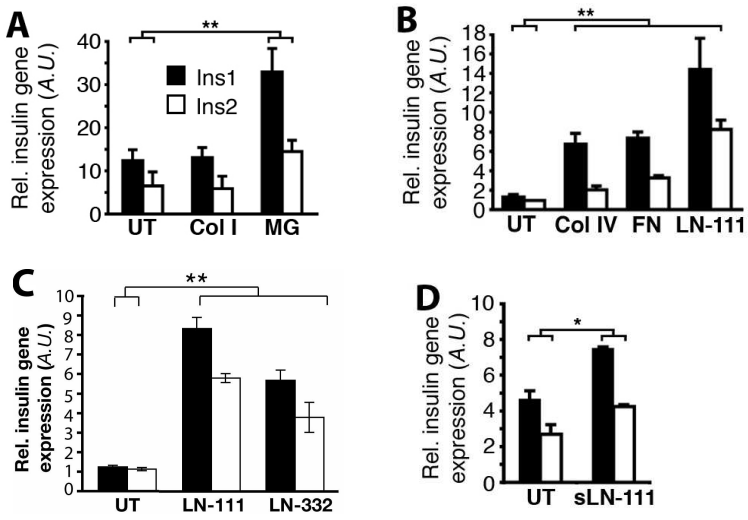

Figure 12. Effect of basement membrane proteins on insulin gene expression. (A-C) Relative gene expression levels of insulin 1 (black bars) and insulin 2 (white bars) in MIN6 cells cultured on different substrates: collagen I matrix (Col I), matrigel (MG), plates coated with collagen IV (Col IV) (5 μg/ml), fibronectin (FN) (5 μg/ml), laminin-111 (LN-111) (5 μg/ml – 12B and 1 μg/ml – 12C), and laminin-322 (LN-322) (1 μg/ml) as well as untreated plates (UT) (n = 3). (D) Relative insulin gene expression in MIN6 cells cultured in media supplemented with 30 μg/ml soluble laminin-111 (sLN-111) (n = 2). *$p < 0.05$, **$p < 0.005$ (Student's t-test). All values are means ± SD.

Most experiments described in this study used laminin-111 as the prototype of laminins, which was available in amounts sufficient for the experiments described here. To investigate whether the up-regulation of insulin gene expression in beta cells was also a feature of the laminins found in the vascular basement membrane, experiments with recombinant laminin-411 (Kortesmaa et al., 2000) and recombinant laminin-511 (Doi et al., 2002) were performed. These laminins contained the laminin α-chains α4 and α5, which were identified in the islet endothelial cells (Figures 8-9). The experiments demonstrated that all laminins up-regulated insulin gene expression, but did not affect the expression of two control genes, β2-microglobulin and cyclophilin (Figures 13A – 13C).

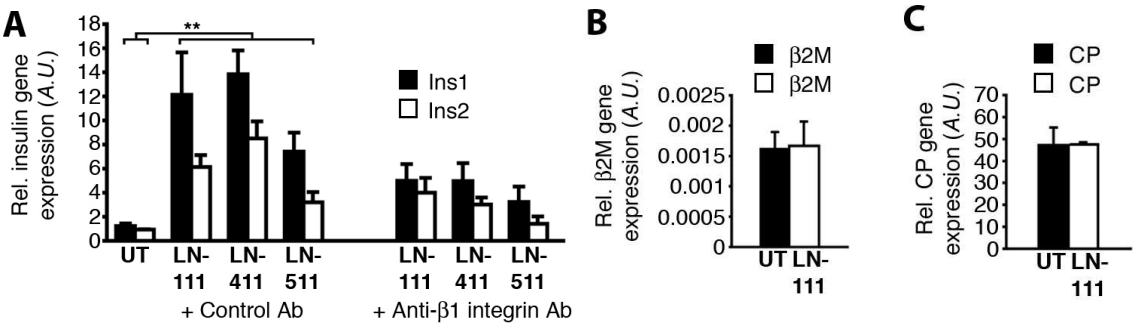

Figure 13. **The effect on laminins on insulin gene expression can be inhibited with blocking antibody against β1-integrin**. (A) Relative insulin gene expression in MIN6 cells cultured on plates coated with laminins-111 (LN-111), -411 (LN-411) and -511 (LN-511). The culture media were supplemented with either control antibody or anti-β1 integrin blocking antibody (n = 2). (B, C) Relative expression of the control genes β2-microglobulin (β2M) and cyclophilin (CP) in MIN6 cells plated on untreated (UT) or laminin-treated plates (LN-111) (n = 3). **p < 0.005 (Student's t-test). All values are means ± SD.

Treatment of VEGF-A deficient mutant islets, with a combination of basement membrane proteins, partially rescued physiologic insulin gene expression as defined by control islets (Figure 14A). Moreover, laminin-111, as well as the vascular laminins (laminin-411 and -511), were able to up-regulate insulin gene expression in mutant islets (Figure 14B). Similar

to MIN6 cells, the effect of these laminins was inhibited by using anti-β1-integrin blocking antibodies (Figures 13A and 14B). Insulin gene expression could also be increased in control islets, but to a lesser extent than in mutant islets (Figure 14A). The conclusion from this experiment is that laminins, among other proteins of the vascular basement membrane, act as endothelial signals, which increase insulin gene expression in beta cells.

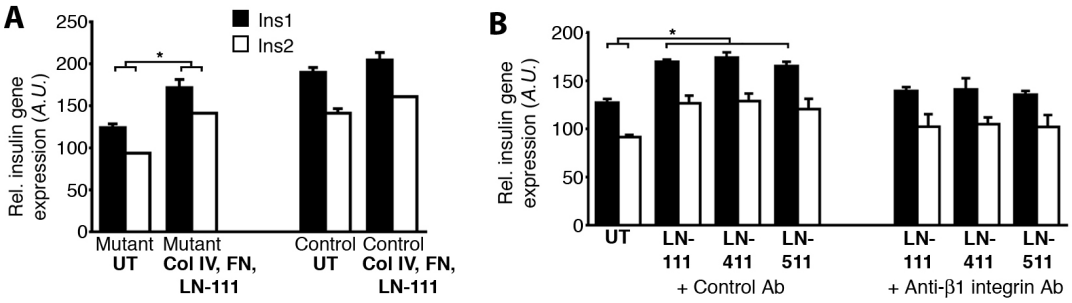

Figure 14. **Insulin-gene expression levels can be partially rescued when mutant islets are treated with basement membrane proteins.** (A) Relative insulin gene expression in mutant and control islets treated with media only (UT) or media supplemented with soluble basement membrane proteins (Col IV, FN, LN-111) (n = 2). (B) Relative insulin gene expression in mutant islets treated with LN-111, LN-411 and LN-511 (n = 2). Islets were cultured in the presence of either control antibody or anti-β1 integrin blocking antibody. *$p < 0.05$ (Student's t-test). All values are means ± SD.

9. β1-integrin is required for up-regulating insulin gene expression in response to laminins

Next I investigated whether the β1-integrin subunit, which forms laminin receptors with various α-integrin subunits, was required for the beta cell response to laminins. Gene expression analyses of all integrin subunits revealed that MIN6 cells and pancreatic islets expressed the mRNA of β1-integrin along with several other β- and α- subunits (Figure 15A). Also, the β1-integrin was localized on the plasma membranes of both MIN6 cells (Figure 15B) and pancreatic islet cells (Figure 15D). High-pressure perfusion of mice was then applied to separate the endothelial cells from beta cells and show that the β1-integrin

was localized on the beta cell plasma membrane, which faced the endothelial cell-derived basement membrane (Figure 15E). Thus, based on its expression and localization, β1-integrin on beta cells could directly interact with the vascular basement membrane.

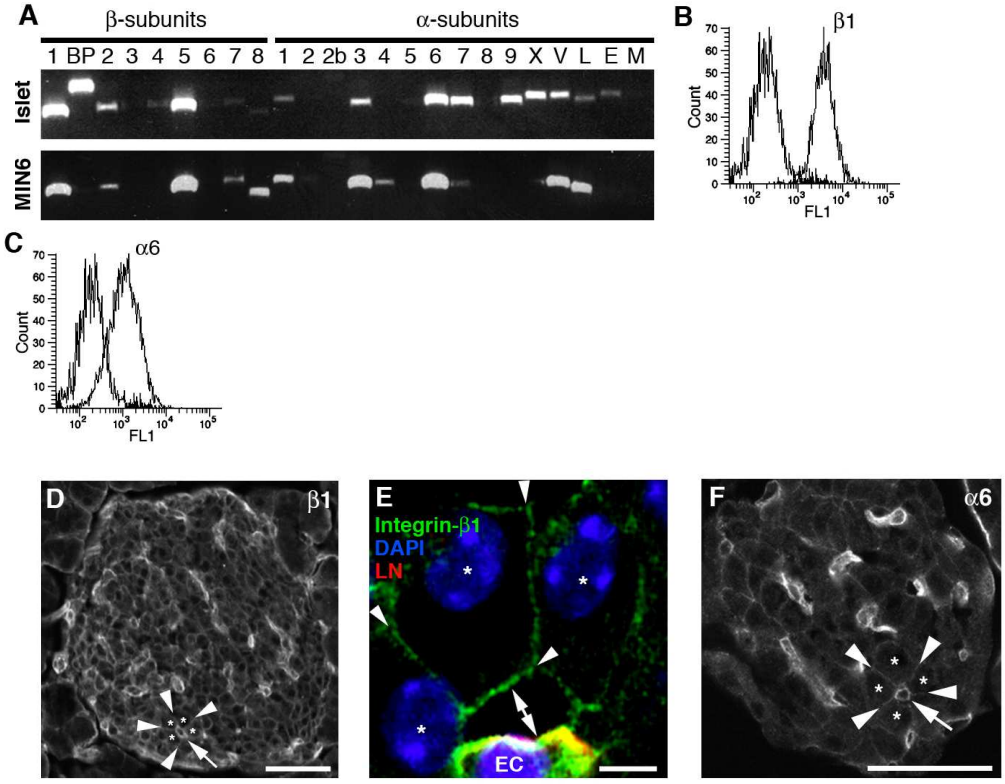

Figure 15. β1- and α6- integrin expression in MIN6 cells and pancreatic islets. (A) Gene expression of integrin β and α subunits in islets and MIN6 cells as demonstrated by RT-PCR. (B, C) FACS histograms of MIN6 cells: unstained and stained for the integrin subunits β1 and α6. (D-F) Confocal images of mouse pancreas sections stained for β1 (D, E) and α6 (F). Capillaries are labeled with arrows (D, F) and beta cell plasma membranes with arrowheads. Beta cells (labeled with asterisks) are often grouped as rosettes around capillaries. The artificial space (↔) between a capillary endothelial cell (EC) and beta cell in (E) was generated by high-pressure perfusion to illustrate β1-integrin expression on the beta cell plasma membrane facing the LN-expressing EC. Scale bars in D, F, 50 μm and in E, 5 μm.

Two strategies were used to investigate if this integrin was required for responding to laminins. First, I used an anti-β1-integrin blocking antibody in MIN6 cell culture, as well as pancreatic islet culture, to show that β1-integrin signaling was required for the beta cell

response to laminins (Figures 13A and 14B). Second, the β1-integrin mRNA level was decreased by 80 to 90% by using two different sets of siRNA molecules (Figures 16A and 16E). This resulted in a 60 to 80% reduced β1-integrin cell surface expression (Figures 16C and 16F). Using this approach, I found that β1-integrin was strictly required for up-regulating insulin gene expression in response to laminin-111 (Figures 16D and 16G). In contrast, it was not required for insulin gene expression in untreated cells (compare UT with LN-111 in Figures 16D and 16G).

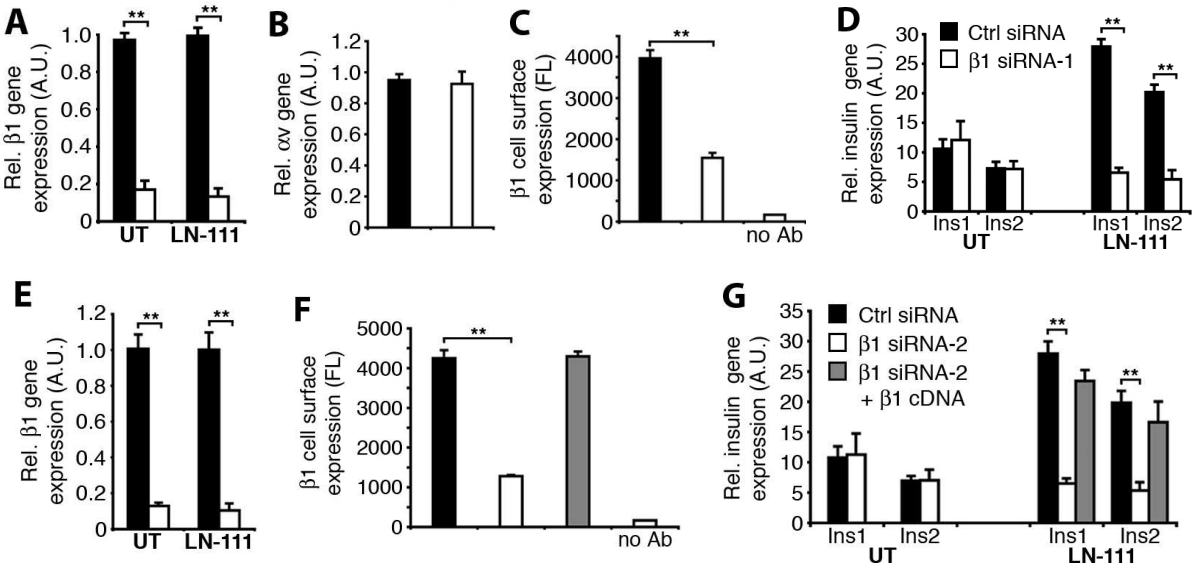

Figure 16. **β1-integrin is required for upregulation of insulin gene expression in MIN6 cells in response to laminin-111.** (A-G) β1-integrin knockdown experiments. The black bars represent MIN6 cells transfected with Ctrl siRNA, the white bars represent MIN6 cells transfected with either β1 siRNA-1 (A-D) or β1 siRNA-2 (E-G), and the grey bars represent β1 siRNA-2 transfected MIN6 cells rescued by the β1 cDNA. (A, E) β1-integrin gene expression in MIN6 cells cultured on untreated plates (UT) and laminin-coated plates (LN-111). (B) Integrin αV gene expression in β1 siRNA-1 transfected MIN6 cells. (C, F) β1-integrin cell surface expression; FL, mean fluorescence. (D, G) Insulin (Ins1 and Ins2) gene expression in MIN6 cells grown on untreated (UT) and laminin-coated plates (LN-111). **p < 0.005 (Student's t-test). N = 3 for each experiment. All values are means ± SD.

10. α6- integrin is also implicated in regulating insulin gene expression

Because of the possible role of the α6β1-integrin in insulin secretion (Bosco et al., 2000), the expression and localization of the α6-integrin was also studied. We found that α6-integrin was localized on the plasma membranes of MIN6 cells (Figure 15C) and islet beta cells (Figure 15F), similar to the localization of β1-integrin (Figures 15B, 15D and 15E). Knockdown of α6-integrin by using two different sets of siRNA molecules decreased the response of MIN6 cells to laminin-111 (Figures 17D and 17G).

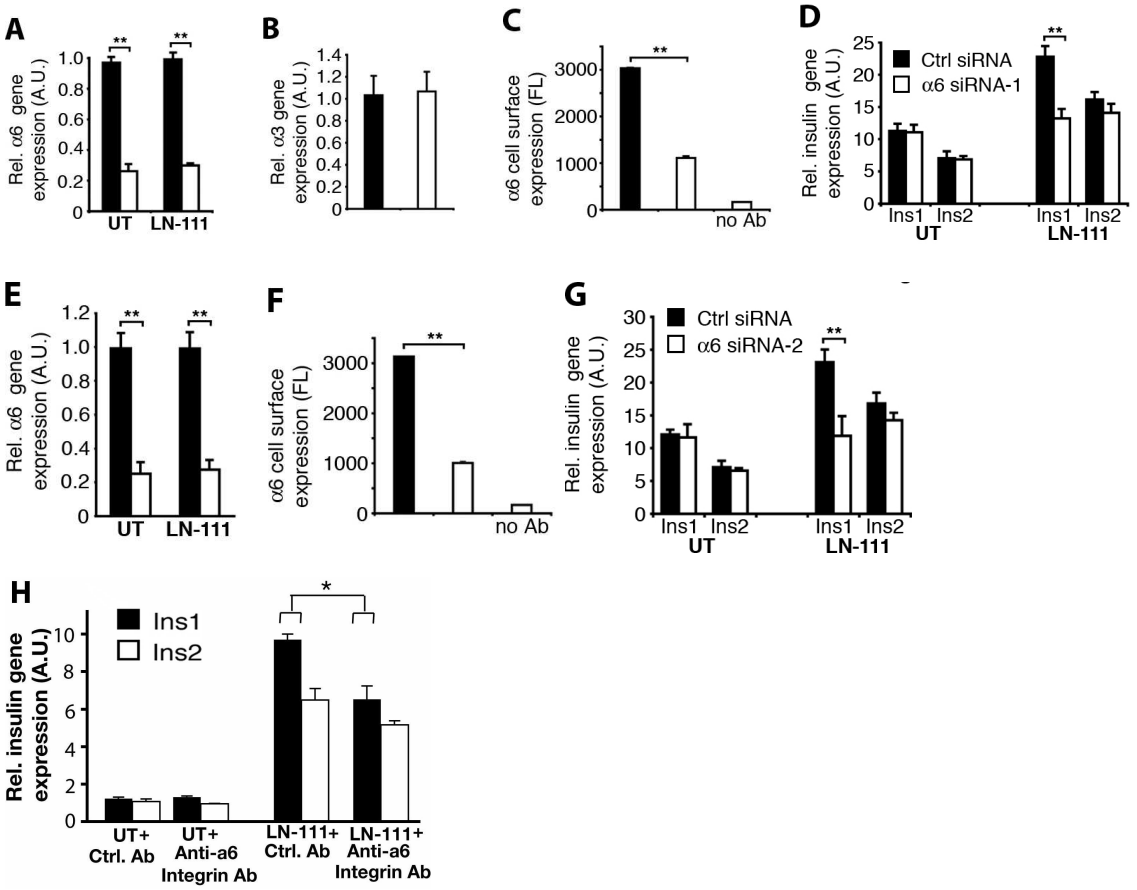

Figure 17. **α6-integrin is also involved in regulation of insulin gene expression.** (A-G) α6-integrin knockdown experiments. The black bars represent MIN6 cells transfected with Ctrl siRNA and the white bars represent MIN6 cells transfected with either α6 siRNA-1 (A-D) or α6 siRNA-2 (E-G). (A, E) α6-integrin gene expression in MIN6 cells cultured on untreated plates (UT) and laminin-coated plates (LN-111). (B) α3-integrin gene expression in α6 siRNA-1 transfected MIN6 cells. (E, F) α6-integrin cell surface expression; FL, mean fluorescence. (D, G) Insulin (Ins1 and Ins2) gene expression in MIN6 cells grown on untreated (UT) and laminin-coated plates (LN-111). (H) Relative insulin gene expression levels (ins1 and ins2) in MIN6 cells cultured on plates coated with laminin-111 (LN-111). MIN6 cells were incubated with either control or anti-α6 integrin blocking antibody for 40 min and then plated on LN-111 coated plates (N=2).

**p < 0.005 (Student's t-test). N = 3 for each experiment. All values are means ± SD.

However, in contrast to the β1-integrin-knockdown, α6-integrin knockdown and also anti-α6-integrin blocking antibody only partially impaired this response (compare Figures 17D and 17G with Figures 16D and 16G; Figure 17H with 13A). This difference might be due to

the presence of other α-subunits in beta cells (Figure15A) that combine with β1-integrin to form alternative laminin receptors.

The specificity of β1- and α6-integrin knockdowns was confirmed by using two sets of siRNA molecules, siRNA-1 and siRNA-2, for each gene, which led to the same biological effects (Figures 16D and 16G, 17D and 17G). In addition, I showed that the knockdown of β1-integrin did not affect the expression of other integrin subunits such as αV and α3, which share sequence homology with β1 and α3 accordingly (Figures 16B and 17B). Lastly, the response to laminin-111 could be restored in β1-integrin-silenced cells by transfection with a β1-integrin cDNA, in which the targeted 3'-UTR was missing (Figures 16F and 16G) (Wennerberg et al., 1996). Along with the experiments using blocking antibodies (Figures 13A, 14B and 17H), the results of the knockdown experiments demonstrate that beta cells require β1-integrin and also α6-integrin for up-regulating insulin gene expression in response to laminins.

The effect of laminins on insulin gene expression could be mimicked by antibody-mediated integrin clustering (Figure 18) although the insulin upregulation was less strong compared MIN6 cells grown on basement membrane protein components (Figure 12A – 12C).

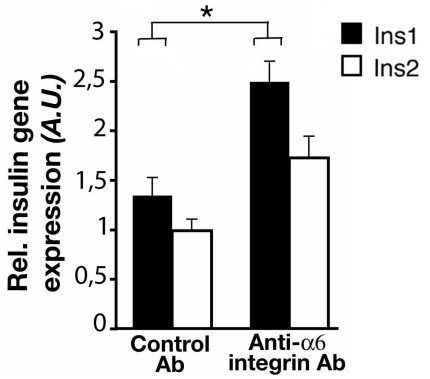

Figure 18. **Antibody-mediated integrin clustering can mimick the effect of laminins on insulin gene expression.** Relative insulin gene expression levels (Ins1 and Ins2) in MIN6 cells grown on plates precoated with anti-rat IgG (secondary Ab) followed by coating with either control antibody or antibody against integrin α6 (primary Ab). *$p < 0.05$ (Student's t-test); N = 2. All values are means ± SD.

11. Laminins regulate pancreatic beta cell proliferation and require β1-integrin

Because the beta cell mass correlates with the vascular density of islets (Duvillie et al., 2002; Inoue et al., 2002; Lammert et al., 2003), the question was raised as to whether endothelial cells regulate beta cell proliferation (Zaret, 2004). The number of MIN6 cells in S phase increased when they were cultured on plates coated with basement membrane proteins, with the strongest effect induced by laminin-111 (Figure 19A). In addition, RNA interference showed that β1-integrin was required for the increased cell proliferation rate induced by this laminin (Figure 19B). Moreover, all laminins tested (laminins -111, -411 and -511) increased MIN6 cell proliferation, and anti-β1 integrin blocking antibodies extinguished this proliferative effect (Figure 19C).

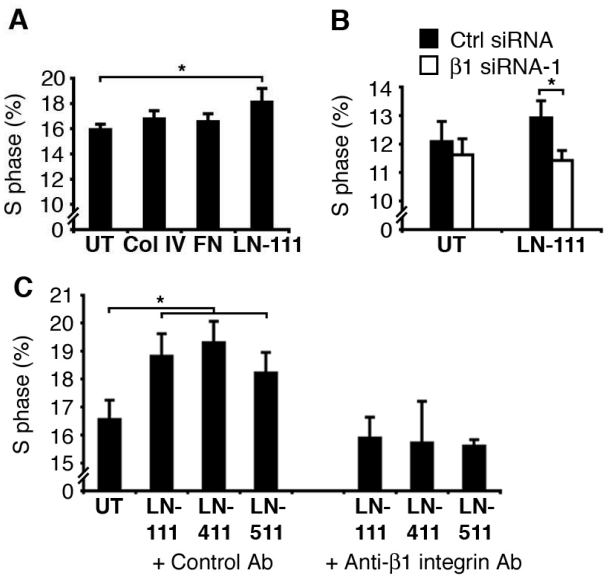

Figure 19. **Regulation of MIN6 beta cell proliferation by laminins and requirement for β1-integrin.** (A) MIN6 cell proliferation in response to collagen IV (Col IV), fibronectin (FN) and laminin-111 (LN-111) compared with untreated cells (UT) (n = 3). (B) Percentage of MIN6 cells in S phase after transfection with β1 siRNA-1 (white bars) and Ctrl siRNA (black bars). Cells were plated on untreated plates (UT) and laminin-coated plates (LN-111) (n = 4). (C) Percentage of MIN6 cells in S phase after plating on LN-111, LN-411 and LN-511. Cells were cultured in the presence of control antibody or anti-β1 integrin blocking antibody (n = 3). *$p < 0.05$ (Student's t-test). All values are means ± SD. Note that the ordinates in A to C were abbreviated.

To extend these findings to mouse islets, beta cell proliferation rates were compared between VEGF-A deficient mutant islets and control islets (Figure 20A). Mutant islets were smaller on average (compare Figures 7B, D with Figures 7A, C) (Lammert et al., 2003) and had fewer proliferating beta cells than control islets (Figure 20B, compare the first two columns). Importantly, beta cell proliferation was partially rescued when mutant islets were treated with laminin-111 (Figure 20B, compare third column with fourth column). The beta cell proliferation rate also increased in control islets, but to a lesser extent when compared with mutant islets (Figures 20B). In addition, treatment of mutant islets with an anti-β1 integrin blocking antibody reduced the proliferative response of islet beta cells to laminin-111 (Figure 20C).

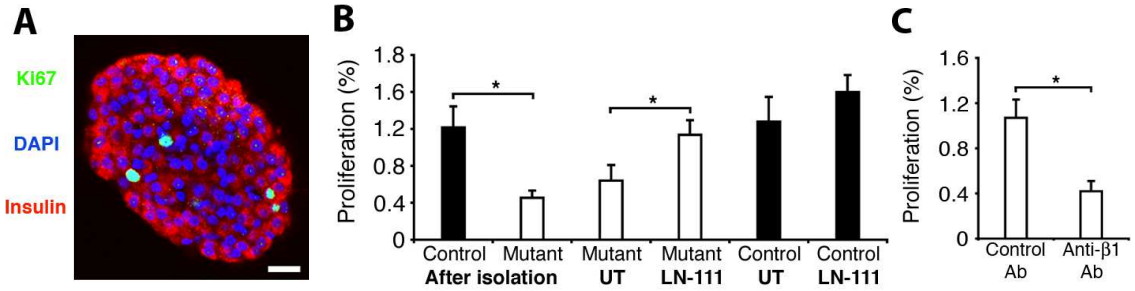

Figure 20. **Beta cell proliferation defect in mutant islets and rescue by laminin-111.** (A) Isolated islets were stained for Ki67 (green), insulin (red) and nuclei (blue). Cells co-expressing Ki67 and insulin were counted as proliferating beta cells. Scale bar, 20 μm. (B) Beta cell proliferation (as percentage of Ki67 positive beta cells in the total islet cell population) was determined in control islets (black bars) and mutant islets (white bars) immediately after islet isolation as well as 3 days after incubation without (UT) and with laminin-111 (LN-111). (C) Beta cell proliferation was determined in mutant islets 3 days after incubation with laminin-111 in the presence of either control antibody or anti-β1 integrin blocking antibody.

Numbers of islets analyzed ($Ki67^+$cells/total cells counted): 12 control islets (92/7,896) and 17 mutant islets (49/10,525) after isolation; 8 untreated mutant islets (44/6,443) and 11 LN-111-treated mutant islets (103/9,136); 11 untreated control islets (88/6,430) and 17 LN-111-treated control islets (214/13,321); 12 LN-111-treated mutant islets with control antibody (90/8,344) and 10 LN-111-treated mutant islets with anti-β1 integrin blocking antibody (39/9,013).

*$p < 0.05$ (Student's t-test). Values in (B and C) are means ± SEM.

These results show that the laminins are signals from the vascular basement membrane, which stimulate beta cell proliferation. In addition, the proliferative effect of the laminins requires β1-integrin at beta cell plasma membrane.

PROPOSED MODEL. CONCLUSION AND PERSPECTIVES

Based on the obtained results, the following model could be proposed (Figure 21): Beta cells do not form a basement membrane. Instead, by using VEGF-A, they attract endothelial cells, which in turn form capillaries with a vascular basement membrane next to the beta cells. Laminins, among other basement membrane proteins, act as signals from the vascular basement membrane, which increase beta cell proliferation and insulin gene expression. β1-integrin on beta cells is required for these effects.

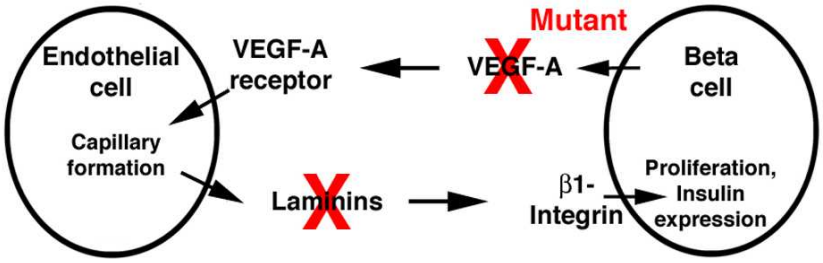

Figure 21.Proposed model (see the text).

The following evidence provides support for this model:

Pancreatic beta cells express neither laminin α-chains nor collagen IV chains α1 and α2 (Figure 9). Because the presence of either laminin α1 or α5 is required for basement membrane formation (Miner et al., 2004; Yurchenco et al., 1997; Yurchenco and Wadsworth, 2004), beta cells cannot produce their own basement membrane. Also , as shown in Figure 9, unlike beta cells, islet endothelial cells expressed the laminin chains α4 and α5 as well as collagen IV chains α1 and α2.

By using mice with a pancreas specific VEGF-A deletion (Lammert et al., 2003), I demonstrated that the loss of islet VEGF-A resulted in a loss of the intra-islet basement membrane, coincident with the loss of endothelial cells (Figures 7A to 7D). Thus, beta cells

secreted VEGF-A to attract endothelial cells, which then formed a basement membrane next to the beta cells.

I also found that islets with no intra-islet basement membrane had decreased insulin gene expression levels and reduced numbers of insulin secretory granules (Figures 10 and11).

To see whether the vascular basement membrane harbored signals that stimulated insulin gene expression, single basement membrane proteins were tested on MIN6 cells. Using these cells, the laminins were identified as endothelial signals that were capable of up-regulating insulin gene expression (Figure 12B - 12D and 13A). The basement membrane proteins, collagen IV and fibronectin, also increased insulin gene expression (Figure 12B), in contrast to the interstitial collagen I, which had no effect on insulin expression (Figure 12A). The observation that non-polymerizing laminin-8 could also stimulate insulin gene expression in beta cells suggested that formation of a resilient laminin substrate was not required (Figures 13A and 14B). In addition, single laminins, similar to a mixture of basement membrane proteins, were able to partially rescue physiological levels of insulin gene expression in VEGF-A deficient islets (Figures 14A and 14B). Thus, laminins and some other protein components of the vascular basement membrane, stimulate insulin gene expression in beta cells.

It was recently described that VEGF-A deficient islets had a smaller size than the control islets (compare Figures 7B, D with 7A, C) (Lammert et al., 2003), suggesting that endothelial cells were involved in stimulating beta cell proliferation (Zaret, 2004).

Work on the embryonic pancreas demonstrated that vascular endothelium stimulated dorsal pancreatic growth (Lammert et al., 2001; Yoshitomi and Zaret, 2004). The question of whether endothelial cells also promoted adult beta cell proliferation was not addressed in our previous reports (Lammert et al., 2001; Lammert et al., 2003). It turned out that beta cells in VEGF-A deficient islets had reduced proliferation rate (Figure 20B, first two columns). I then showed that laminins, among other proteins of the vascular basement membrane, stimulated

proliferation in MIN6 cells (Figures 19A to 19C). In addition, laminin-1 was able to partially rescue beta cell proliferation in VEGF-A deficient islets (Figure 20B, third and fourth columns). These results suggested that laminins contributed to the beta cell proliferation rate in pancreatic islets.

Next the question was raised as to whether β1-integrin was required for the response to laminins. High-pressure perfusion of mice showed that β1-integrin was localized on the beta cell plasma membrane adjacent to the vascular basement membrane (Figure 15E). Thus, β1-integrin location allowed beta cells to interact with the vascular basement membrane. The α6-integrin subunit, which was previously shown to form a heterodimer with the β1-integrin subunit in beta cells (Bosco et al., 2000), was similarly present on the beta cell plasma membrane (Figure 15F). I then demonstrated that β1-integrin was required for promoting insulin gene expression and proliferation in response to laminins by using anti-β1-integrin blocking antibodies (Figures 13A and 14B; 19C and 20C) as well as RNA interference (Figures 16D,G; 19B). The presence of the α6β1-heterodimer in beta cells (Bosco et al., 2000), the location of β1-integrin and α6-integrin in MIN6 cells (Figures 15A-15C) as well as pancreatic islet cells (Figures 15D-15F), the effect of an anti-α6 integrin blocking antibody ((Figure 17H) and the results of the α6-integrin knockdown (Figures 17A-17G) suggested that the α6β1-integrin was a laminin receptor on beta cells involved in regulating insulin gene expression.

It is well known that the binding of an integrin to its ligand induces aggregation of integrins on the plasma membrane which is necessary for effective signal transduction (Brinkerhoff and Linderman, 2005, Hynes RO, 2002). The importance of integrin clustering can be also demonstrated by nonphysiological techniques such as chemical cross-linking agents or antibodies (Hato et al., 1998). The observed upregulation of insulin gene expression after antibody mediated integrin clustering (Figure 18) could be possibly due to integrin dimerization which is necessary for initiation of integrin signaling. However, it is known that

in general, the strength of the effect that integrin dimerization alone by itself can induce, varies depending on the cell type and the antibody used (Wei et al., 1998, Chen et al., 2005, Liu et al., 2005). It is also known that ligand presence and organization together with integrin dimerization (in this case induced by ligand presence) cooperate to increase both binding and clustering of integrins and in this way leading to an optimal biological effect (Brinkerhoff and Linderman, 2005).

In this study, a combination of *in vivo*, *ex vivo* and *in vitro* experiments was used to investigate the interaction of adult beta cells with capillary endothelial cells. Laminins and β1-integrin were considered as the molecular players involved in this cellular interaction. The results of the experiments suggested the model outlined in Figure 21. A complete deletion of the vascular basement membrane or pancreatic β1-integrin in mice would have resulted in diverse embryonic phenotypes, and therefore, made it difficult to study their roles in the adult islet (Kaido et al., 2004; Thyboll et al., 2002). Thus, to identify endothelial signals acting on adult beta cells, rescue experiments were performed. The components of the basement membrane were added to the culture media of adult VEGF-A deficient mouse islets (Figures 14A, 14B and 20B). These mutant islets had few endothelial cells, and some islets completely lacked endothelial cells (Figures 7B and 7D). Thus, in these mutant islets, many beta cells were not adjacent to a basement membrane and their function could therefore be rescued by components of the vascular basement membrane. It is noteworthy that the islet capsule represents an alternative, non-vascular source of laminin and collagen IV (Figures 7B and 7D). However, because of the spheroid structure of islets, most beta cells are not in contact with the islet capsule and therefore require a vascular basement membrane.

The proposed model for this study (Figure 21) provides an explanation for the following observations: first, pancreatic islets, like other endocrine glands, express high levels of VEGF (Inoue et al., 2002; Lammert et al., 2001; LeCouter et al., 2001); second, islets harbor a capillary network that is five times denser than that of the exocrine pancreatic tissue and

therefore exceeds the general tissue requirement for vascular supply (Henderson and Moss, 1985); and third, over-expression of VEGF-A in islets makes their transplantation more successful in terms of curing diabetes mellitus (Zhang et al., 2004). A practical aspect of this model is to use basement membrane proteins as *in vitro* substitutes for endothelial cells to improve islet cell function and proliferation. Research on islet transplantation has shown that it takes about 1-2 weeks for transplanted islets to become re-vascularized in the host (Jansson and Carlsson, 2002). It has been suggested that many islets lose their endocrine function during this time frame, explaining why high numbers of islets are needed for transplantation (Jansson and Carlsson, 2002). Based on this study, examination of whether the treatment of islets with laminin-111 improves islet transplantation is warranted. Laminin-111, in contrast to the other laminins (laminin-411 and -511), is easily available in unlimited amounts (Timpl et al., 1979). The results presented in the current study suggest that laminin-111 can partially substitute for endothelial cells and may help to bridge the time until new capillaries and vascular basement membranes are formed in transplanted islets.

Finally, it is likely that the principal components of the vascular basement membrane, as permissive signals, act in concert with instructive and cell-type specific signals. Thus, the vascular basement membrane might be involved in various processes, in which endothelial cells play a critical role, including liver morphogenesis and growth (LeCouter et al., 2003; Matsumoto et al., 2001), neural stem cell proliferation, differentiation and transdifferentiation (Palmer et al., 2000; Shen et al., 2004; Wurmser et al., 2004), as well as cancer growth and metastasis (Hanahan and Folkman, 1996; Ruoslahti, 2002; Sipkins et al., 2005).

MATERIALS AND METHODS

Laminin nomenclature

Previously, laminin trimers were numbered with Arabic numbers in the order they were discovered: that is laminins-1 to -15. Aumailley et al., introduced a new identification system for a trimer using three Arabic numerals, based on the α, β and γ chain numbers (Aumailley et al., 2005). According to the previous nomenclature (Burgeson et al., 1994), a trimer could be identified either by the Arabic numeral (e.g., -10), or by its chains (e.g., α5β1γ1). An abbreviated form, 511 for α5β1γ1, which stands for laminin-10, can equally well be used with no loss of information. 511 is more informative than laminin-10 and obviates the need to memorize the chain composition. Therefore, according to the new nomenclature laminin trimers are named solely on the basis of the chain composition, either by mentioning (Table 1) or not mentioning the Greek letters.

Cell culture

The MIN6 insulinoma cell line (Miyazaki et al., 1990) was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 22 mM glucose (Gibco), supplemented with 15% fetal calf serum (FCS) (PAA), 100 U/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco), 70 µM β-mercaptoethanol (Gibco), 1.5 g/L $NaHCO_3$ (Gibco), in a humidified atmosphere (5% CO_2, 37°C). All MIN6 cells used in this work were at passages 39 – 46. Mouse laminin (Beckton Dickinson), recombinant laminin-411 (LN-411) (Kortesmaa et al., 2000), recombinant laminin-511 (LN-511) (Doi et al., 2002), laminin 511/521 (LN511/521) (a gift from L.Sorokin), mouse collagen IV (Beckton Dickinson), human fibronectin (Upstate) were used at a concentration of 5 µg/ml for coating non-tissue culture treated 6-well plates (Falcon) according to the manufacturers' instructions. When the effect of LN-111

was compared with Laminin-322 (Chemicon), both laminins were used at concentration of 1 μg/ml. When laminin was added to culture media, it was used at 30 μg/ml. Collagen type I (Beckton Dickinson) as well as growth factor deprived matrigel (BD Pharmingen) were used as 5 mm thick gels. Hamster IgM and hamster CD29 IgM (Ha2/5) (Beckton Dickinson) were used as control and anti-β1-integrin blocking antibodies at concentration 30 μg/ml when insulin gene expression was investigated and 45 μg/ml for S-phase analysis. Rat Ig_{2a} and rat anti-human CD49f (BD Pharmingen) were used as control and anti-α6-integrin blocking/activating antibodies at concentration 20 μg/ml. All plates were blocked with 1% BSA (Sigma). Untreated wells (UT) were not coated, but blocked with 1% BSA. An integrin-crosslinking experiment was performed using Rat Ig_{2a}, rat anti-human CD49f (BD Pharmingen) and donkey anti-rat-IgG (Molecular probes). The antibodies were used at concentration10 μg/ml. Non-tissue culture treated 6-well plates (Falcon) were first coated with donkey anti-rat-IgG overnight at 4 C°. After washing with PBS, the plates were incubated with rat Ig_{2a} and rat anti-human CD49f at room temperature for 2. Cells were incubated for 3-4 days after setting up each assay.

Table 1. Nomenclature of 16 laminins (Aumailley et al., 2005)

Standard	Abbreviated	Previous
α1β1γ1	111	1
α2β1γ1	211	2
α1β2γ1	121	3
α2β2γ1	221	4
α3Aβ3γ2	332, or 3A32	5, or 5A
α3Bβ3γ2	3B32	5B
α3Aβ1γ1	311, or 3A11	6, or 6A
α3Aβ2γ1	321, or 3A21	7, or 7A
α4β1γ1	411	8
α4β2γ1	421	9
α5β1γ1	511	10
α5β2γ1	521	11
α2β1γ3	213	12
α4β2γ3	423	14
α5β2γ2	522	-
α5β2γ3	523	15

Pancreatic islet culture

Pancreatic islets isolated from mutant and control mice were cultured for 3 days in DMEM containing 22 mM glucose (Gibco), supplemented with 15% fetal calf serum (FCS) (PAA), 100 U/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco), 70 µM β-mercaptoethanol (Gibco), 1.5 g/L $NaHCO_3$ (Gibco) in a humidified atmosphere (5% CO_2, 37°C). Islets were cultured in medium alone (UT) or medium containing 30 µg/ml soluble laminin (Beckton Dickinson), 10 µg/ml collagen IV (Beckton Dickinson) and10 µg/ml fibronectin (Upstate) (Col IV, FN, LN) or 30 µg/ml LN-111, LN-411, or LN-511. The mRNAs were isolated either directly after islet isolation or after 3 days incubation. For assessment of beta cell proliferation, islets were cultured for 3 days in medium alone (UT) or medium containing 50 µg/ml soluble laminin (LN) with either 45 µg/ml hamster IgM (control Ab) or 45 µg/ml hamster anti-Rat CD29 IgM (Ha2/5) (anti-b1-integrin blocking Ab). The culture medium was changed after 1.5 days.

Cell transfection with siRNA

The Amaxa electroporation system was used to transfect MIN6 cells (program G16) according to the manufacturers' instructions (Amaxa biosystems). MIN6 cells were transfected with 5 µg siRNA molecules directed against integrin subunits β1 (β1 siRNA-1 and β1 siRNA-2) and α6 (α6 siRNA-1 and α6 siRNA-2) as well as firefly luciferase (Ctrl siRNA). The following T7-tagged primer pairs (MWG) were used for siRNA synthesis:
β1 siRNA-1: 5'-ATTAATACGACTCACTATAGGTCTGGAAAATTCTGCGAGTG-3' and 5'-CGTAATACGACTCACTATAGGCAACTGTGGTCAATCCGAAG-3';

β1 siRNA-2: 5'-ATTAATACGACTCACTATAGGTAATCCATGCCAGGGACTGAC-3' and

5'-CGTAATACGACTCACTATAGGCCCACCTCTCAGAATTTTTCC-3'

α6 siRNA-1: 5'-ATTAATACGACTCACTATAGGCGCAGGTGTATTTTGGAGG-3' and 5'-CGTAATACGACTCACTATAGGGATGCCACCTATCACAAGGC-3';

α6 siRNA-2: 5'-ATTAATACGACTCACTATAGGACCGAGGTCACCTTTGACAC-3' and 5'-CGTAATACGACTCACTATAGGTTTTTCAGGAAGTTCCCGTTT-3'

The specificity of each siRNA sequence was checked by using the DEQOR program (Henschel et al., 2004). After siRNA transfection, MIN6 cells were plated on non-tissue culture treated plates and grown for 36 h. Transfected cells were transferred to laminin coated (LN) and untreated (UT) plates and incubated for another 72 hours. Cells were then harvested for RNA isolation and cDNA synthesis.

RNA isolation, reverse transcription and conventional PCR

Total RNAs from MIN6 cells and pancreatic islets were extracted using the RNeasy Mini kit (Qiagen) including Dnase I digest (Qiagen). For every experiment, 1 μg total RNA from MIN6 cells and 500 ng total RNA from islets were used for reverse transcription. cDNA was synthesized using MMLV-RT (Promega) and Oligo $(dT)_{15}$ primer (Promega) with controls lacking reverse transcriptase (-RT). The cDNA was used for conventional PCR or quantitative real-time RT-PCR.

The conventional PCR for detection of integrin subunits in MIN6 cells and mouse islets was performed with a PTC-200 thermal cycler (MJ Research, USA). An initial denaturation step for 5 min was followed by 39 cycles of annealing at 56°C for 30 sec., elongation at 72°C for

1.5 min, and denaturation at 94°C for 30 sec. PCR reactions were performed in a 20 µl reaction volume using 1 µl cDNA and 1 U Taq DNA polymerase (Promega). The RT-PCR products were run on a 1% agarose gel. All integrin primers were first tested using cDNA pooled from different mouse embryonic and adult organs (positive controls), and only primers, which gave the expected PCR product in the positive controls were used.

Generation of siRNA

Small interfering RNAs (siRNA) were prepared according to Kittler et al., (2004). cDNA templates were PCR amplified using T7 – appended forward and reverse primers. PCR products were analyzed on a 1,5% (wt/vol) agarose gel. Only PCR products which gave high yield of amplification (as indicated from intense PCR bands) were used for in-vitro transcription reaction. In- vitro transcription reaction (20 µl volume) was assembled using MEGAscript kit (Ambion): 2 µl ATP, 2 µl CTP, 2 µl GTP, 2 µl UTP, 2 µl 10x T7 reaction buffer, 8 µl PCR product, 2 µl T7 enzyme mix. In-vitro transcription and annealing reactions were performed in a thermal cycler as follows:

In-vitro transcription	4 h – at 37°C
Denaturation	3 min at 90°C
	Ramp to 70°C with 0,1°C/s
	3 min at 70°C
	Ramp to 50°C with 0,1°C/s
	3 min at 50°C
Annealing	Ramp to 25°C with 0,1°C/s
End	

0,5 µl aliquots from in-vitro transcribed products were run on a 1,5% (wt/vol) agarose gel. The digestion reactions were set up as follows: 20 µl of the in-vitro transcription reaction product were 200 µl of dsRNA digestion buffer, containing 8 µg GST-RNAse III. The mixture was incubated with agitation for 4h at 25 °C. The digestion products were checked on 4% gel. The digestion product smear should be between around 23 bp. Spin columns (Bio Rad) for purification were prepared as follows: 200 µl of Q-Sepharose (Amersham Biosciences) was added to an empty spin column. The column was put on an empty 2-ml microcentrifuge tube. 500 µl of equilibration buffer was added to the column. The columns were centrifuged at 1,000g for 1 min and the flowthrough was discarded. Another 500 µl of equilibration buffer was added, centrifugation at 1,000 for 1 min was performed and the flowthrough was discarded. All of the digested dsRNA was loaded onto the column and incubated for 5 min at 25 °C. Centrifugation at 1,000 for 1 min was performed and the flowthrough was discarded. 500 µl of wash buffer was added to the column, followed by centrifuge at 1,000g for 1 min and discarding the flowthrough. 300 µl of elution buffer was added and centrifugation at 1,000 for 1 min was performed. The flowthrough was collected in new microcentrifuge tube. Another 300 µl were added to the column and centrifugation at 1,000 for 1 min was performed. 500 µl $(CH_3)_2CHOH$ was added to the collected flowthrough, and the mixture was stored at 4°C for 1h. Centrifugation at 16,000 g for 15 min at 4°C was performed. The supernatant was discarded and the pellet was washed twice with 70% (vol/vol) C_2H_5OH. The pellet was dried and dissolved in 50 µl of H_2O. siRNAs were stored at -20°C.

dsRNA digestion buffer: 20mM Tris-HCl, 0.5 mM EDTA, 5 mM MgCl, 1 mM dithiothreitol, 140 mM NaCl, 2,7 mM KCl, 5% (vol/vol) glycerol (pH 7,9)).

Elution buffer: 20 mM Tris-HCl, 1mM EDTA, 520 mM NaCl (pH 8,0).

Equilibration buffer: 20 mM Tris-HCl, 1 mM EDTA, 300 mM NaCl (pH 8,0).

Wash buffer: 20 mM Tris-HCl, 1 mM EDTA, 400 mM NaCl (pH 8,0).

esiRNA loading die: 3 mg/ml Orange G, 5% (wt/vol) Ficoll.

Quantitative Real-time RT-PCR

cDNA templates from MIN6 cells and mouse islets were analyzed for integrin and insulin 1 and 2 gene expression using the SYBR green method (Brilliant SYBR Green QPCR Master Mix, Stratagene) and the Mx3000 Multiplex Quantitative PCR system (Stratagene) according to the manufacturers' protocol. Control and mutant islet mRNAs were isolated directly after islet isolation or 3 days after incubation. The following primer pairs (MWG) were used for real-time PCR:

α-Tubulin: 5'-CTTTGAGCCAGCCAACCAGAT-3' and

5'-TTGATGGTGGCAATGGCAG-3'

Ins1: 5'-CAGCAAGCAGGTCATTGTTTCAAC-3' and

5'-TGGGTGGGTTTGGGCTCC-3'

Ins2: 5'-CCATCAGCAAGCAGGAAGCCTATC-3' and

5'-CCCCACACACCAGGTAGAGAGCG-3'

β1: 5'-AATGCCAAGTGGGACACGGG-3' and

5'-TGACTAAGATGCTGCTGCTGTGAGC-3'

α6: 5'-AGCCTTCAACCTGGACACCCG-3' and

5'-TGCCCCCACAAGCAACAGC-3'

β2M: 5'-GAGCCCAAGACCGTCTACTG-3' and

5'-GCTATTTCTTTCTGCGTGCAT-3'

CP: 5'-GACGCCACTGTCGCTTTTCG-3' and

5'-CTGCTGTCTTTGGAACTTTGTCTGC-3'

α3: 5'-TTCCTGAGAACCAGCATCCCTACC-3' and

5’-GCCACAAGCACCAACCACAGC-3’

αV: 5’-CGGAACAACGAAGCCTTAGCAAG-3’ and

5’-TGAAACGAAGACCAGCGAGCAG-3’

Note that the integrin primers flank a region different from the region targeted by the siRNA molecules.

For each real-time RT-PCR experiment, 4 µl cDNA were used (always corresponding to 80 ng total MIN6 cell RNA and 40 ng total islet RNA). An initial denaturation step for 10 min at 95°C was followed by 40 cycles of 30 s at 95°C, 1 min at 56°C, and 30 s at 72°C. Each sample of three independent experiments with MIN6 cells and two independent experiments with mouse islets were run in duplicate. For each primer pair ‘No template control’ was included. Data were analyzed according to the threshold cycle method (Ct). The internal control was α-tubulin. Comparison of the α-tubulin mRNA levels with cyclophilin and β2-microglobulin mRNA levels in MIN6 cells, as well as control and mutant islets, validated the use of α-tubulin as internal control. β2-microglobulin and cyclophilin gene expression levels were unchanged in control and mutant islets as well as in MIN6 grown on UT or LN1-treated surfaces. The ΔCt values (gene of interest e.g. insulin 1 – α-tubulin as normalizer) for islets as well as MIN6 cells were calculated; the $2^{-\Delta Ct}$ values represent the relative expression levels of the genes of interest. Expression levels of integrin genes in MIN6 cells transfected with integrin-specific siRNA molecules (β1 siRNA-1 and β1 siRNA-2 or α6 siRNA-1 and α6 siRNA-2) and control siRNA were normalized against the expression levels of cells transfected without siRNA.

Cell cycle analysis

MIN6 cells were plated on non-tissue culture treated 6 well plates coated with laminin (LN), collagen (ColIV) and fibronectin (FN) as described before. MIN6 cells grown on uncoated plates were used as controls (UT). For integrin β1 gene silencing experiments, cells were plated on laminin coated and uncoated plates. After 3-4 days cells were harvested using trypsin-EDTA (PAA), centrifuged for 5 min, washed with PBS, resuspended in PBS containing 70% ethanol and kept overnight at 4°C. Before analyses, cells were resuspended in PBS containing 70% ethanol, centrifuged and finally resuspended in PBS containing 25 μg/ml propidium iodide and 6,25 mg/ml RNAse A (Qiagen). Cells were incubated at room temperature for 15 min. The cell cycle phase distribution (%) was measured with the FACScan flow cytometer (BD) and analyzed by using CELLQuest software.

FACS analysis of integrin protein expression

After transfection with control siRNA, siRNAs directed against two different regions of integrin β1- (β1 siRNA-1 and β1 siRNA-2) or α6 - (α6 siRNA-1 and α6 siRNA-2) MIN6 cells were grown on non-tissue cell culture treated 6-well plates. For performing rescue experiment, MIN6 cells were transfected with β1 siRNA-2 and β1 cDNA (p β1-EGFP-N1). After 3 days cells were harvested using trypsin-EDTA (PAA), immunostained with rat α-Integrin β1 (1:50) (Chemicon) or rat α-Integrin α6 (1:100) (BD Pharmingen). Cy5-(Dianova) conjugated donkey antibody was used as secondary antibodies. MIN6 cells stained with no Abs and only with secondary Ab were used as controls. Integrin β1- and α6- protein expression levels were evaluated by measuring the mean fluorescence using FACSAria flow cytometer (BD).

Mouse animal models and pancreas isolation

Mutant mice (Pdx1-Cre x VEGFloxP) contained the Cre recombinase under the Pdx1 promoter (Offield et al., 1996) and had both VEGF-A alleles floxed (Gerber et al., 1999, Lammert et al., 2003). Heterozygous littermates of the same gender were used as controls. NMRI mice were used for the gene expression and immunohistochemical analyses of the laminin α chains and the integrins (Figures 9, 15) as well as islet endothelial and β cell isolation. Islets were isolated from mouse pancreases by using Liberase RI (Roche). Briefly, after killing mice by cervical dislocation, the bile duct was clamped off at its duodenal insertion by using a small bulldog clamp. 2 ml 0.23 mg/ml Liberase RI solution were injected into the bile duct followed by digestion at 37°C for 23 min. After gradient centrifugation, islets were collected from the interphase between Histopaque 1077 (Sigma) and DMEM. Islets were handpicked under a stereomicroscope and used for RNA isolation, immunohistochemistry or tissue culture or islet cell isolation.

In order to separate the islet endothelial and beta cell populations, islets were dissociated with trypsin-EDTA (PAA). Rat anti-PECAM1 antibody (1:50) (BD Pharmingen) and Cy5-conjugated donkey anti-rat secondary antibody ((Dianova) were used for labeling islet endothelial cells. Autofluorescent beta cells and antibody-labeled endothelial cells were sorted with the FACSAria (BD) and both populations were used for conventional RT-PCR.

Immunohistochemistry and confocal microscopy

For immunohistochemistry, excised pancreases were fixed with 4% paraformaldehyde (PFA) at 4°C overnight. After stepwise infiltration in sucrose solutions (9%, 18% and 30%), pancreases were embedded in O.C.T. compound (Tissue-Tek), frozen and sectioned (10 µm thick sections). Cryosections were washed with PBS and blocked with 2% BSA, 0.1 %

Triton-X100 in PBS for 1 hour at room temperature. Sections were incubated with primary antibodies at room temperature for 1 hour. The following primary antibodies were used: rabbit α-Laminin (1:30) (Sigma), rabbit α-Laminin-111 (1:200), rabbit α-Laminin-411) (1:500) (Sixt et al.,), rabbit α-Laminin-511 (1:500), (Sixt et al.,), rabbit α-Collagen IV (1:60) (Chemicon), rat α-PECAM1 (1:50) (BD Pharmingen), rat α-Integrin β1 (1:50) (Chemicon), rat α-Integrin α6 (1:100) (BD Pharmingen). After washing with PBS + 0.1% Triton-X100, sections were incubated at room temperature for 1 hour with secondary donkey antibodies diluted in PBS containing 2% BSA, 0.1% Triton-X100 and 4% normal donkey serum. For double labeling, Alexa 488- (Molecular Probes) and Cy5- (Dianova) conjugated donkey antibodies were used.

For immunohistochemical analysis of islet beta cell replication, islets of control and mutant mice were fixed in 4% PFA at room temperature for 20 min directly after islet isolation. Alternatively, isolated islets were cultured for 3 days with and without laminin and then fixed. Blocking was done in PBS containing 2% BSA and 0.4% Triton-X100. Primary antibodies: rabbit α Ki-67 (1:1000) (Novocastra laboratories Ltd.); guinea pig α-insulin (1:200) (DAKO). Secondary antibodies: Cy3 conjugated donkey α-guinea pig (1:500) (Dianova); Alexa 488 conjugated donkey α-rabbit (1:500) (Molecular Probes). Nuclei were counterstained with 1 µg/ml DAPI (SIGMA). Images from pancreatic sections were acquired by using Zeiss confocal laser scanning systems. Ki67 positive beta cells and DAPI positive nuclei were counted in Z-sections taken from the whole islet. Beta cell proliferation was expressed as percentage of Ki67 positive beta cells in the total islet cell population. Cell proliferation was determined in control islets and mutant islets immediately after islet isolation as well as 3 days after incubation with and without laminin.

Electron Microscopy

After perfusion of mice with 2% glutaraldehyde (EMS), pancreas pieces containing islets were dissected with forceps and stained with uranyl acetate and osmium tetroxide and embedded using the Embed kit (EMS). 70 nm sections were placed on slot grids, stained with uranyl acetate and lead citrate and analyzed using transmission electron microscopy. Quantification of the number of insulin secretory granules, mitochondria and Golgi in mutant and control mice was performed as described (Lucocq, J., 1993).

Statistical Analysis

All the values are expressed as means ± SD except for Fig. 20 B and C, which shows the values as means ± SEM. Statistical significance was determined by using the two-tailed unpaired Student's t-test, and differences were considered to be statistically significant when $p < 0.05$.

ABBREVIATIONS

Ab, Antibody

AMP, Adenosine mono phosphate

BM, Basement membrane

β2M, Beta 2 microglobulin

Col, Collagen

CP, Cyclophilin

EC, Endothelial cells

esiRNA, Endoribonuclease prepared small interfering ribonucleic acid

ECM, extracellular matrix

FAK, Focal adhesion kinase

FACS, fluorescence activated cell sorting

FN, Fibronectin

GLUT2, Glucose transporter-4

GLP-1, Glucagon-like peptide-1

GC, Golgi complexes

GH, Growth hormone

Hnf-1α, Hepatocyte nuclear factor-1α

Ins1, Insulin 1

Ins2, Insulin 2

ICA512, Islet cell autoantigen 512

LN, Laminin

JAK, Janus kinase

MAPK, Mitogen activated protein kinase

MC, Mitochondria

MIN6, Mouse insulinoma 6

NeuroD1/BETA2, Neurogenic differentiation 1 or β-cell E-box transactivator 2

PRL, Prolactin

Pdx-1, Pancreatic duodenal homeobox-1

PI3K, Phosphatidylinositol 3-kinase

PECAM, platelate endothelial cell adhesion molecule

PKA, Protein kinase A

RNA, Ribonucleic acid

RT-PCR, Reverse transcription polymerase chain reaction

SG, Secretory granules

STAT, Signal transducers and activators of transcription

siRNA, Small interfering ribonucleic acid

SD, Standard deviation

SEM, Standard error of the mean

VEGF-A, Vascular endothelial growth factor-A

REFERENCES

Ahlgren, U., Jonsson, J., Jonsson, L., Simu, K., and Edlund, H. (1998). beta-cell-specific inactivation of the mouse Ipf1/Pdx1 gene results in loss of the beta-cell phenotype and maturity onset diabetes. Genes Dev 12, 1763-1768.

Aird, W. C., Edelberg, J. M., Weiler-Guettler, H., Simmons, W. W., Smith, T. W., and Rosenberg, R. D. (1997). Vascular bed-specific expression of an endothelial cell gene is programmed by the tissue microenvironment. J Cell Biol 138, 1117-1124.

Alberts (2002). Molecular Biology of the Cell.

Aumailley, M., Bruckner-Tuderman, L., Carter, W. G., Deutzmann, R., Edgar, D., Ekblom, P., Engel, J., Engvall, E., Hohenester, E., Jones, J. C., et al. (2005). A simplified laminin nomenclature. Matrix Biol 24, 326-332.

Aumailley, M., and Gayraud, B. (1998). Structure and biological activity of the extracellular matrix. J Mol Med 76, 253-265.

Aumailley, M., and Krieg, T. (1996). Laminins: a family of diverse multifunctional molecules of basement membranes. J Invest Dermatol 106, 209-214.

Aumailley, M., and Smyth, N. (1998). The role of laminins in basement membrane function. J Anat 193 (Pt 1), 1-21.

Aviezer, D., Hecht, D., Safran, M., Eisinger, M., David, G., and Yayon, A. (1994). Perlecan, basal lamina proteoglycan, promotes basic fibroblast growth factor-receptor binding, mitogenesis, and angiogenesis. Cell 79, 1005-1013.

Banting, F. G., and Best, C. H. (1990). Pancreatic extracts. 1922. J Lab Clin Med 115, 254-272.

Ben-Shushan, E., Marshak, S., Shoshkes, M., Cerasi, E., and Melloul, D. (2001). A pancreatic beta -cell-specific enhancer in the human PDX-1 gene is regulated by hepatocyte nuclear factor 3beta (HNF-3beta), HNF-1alpha, and SPs transcription factors. J Biol Chem 276, 17533-17540.

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

Bonner-Weir, S., and Sharma, A. (2002). Pancreatic stem cells. J Pathol 197, 519-526.

Bonner-Weir, S., Trent, D. F., and Weir, G. C. (1983). Partial pancreatectomy in the rat and subsequent defect in glucose-induced insulin release. J Clin Invest 71, 1544-1553.

Bosco, D., Meda, P., Halban, P. A., and Rouiller, D. G. (2000). Importance of cell-matrix interactions in rat islet beta-cell secretion in vitro: role of alpha6beta1 integrin. Diabetes 49, 233-243.

Bosman, F. T., and Stamenkovic, I. (2003). Functional structure and composition of the extracellular matrix. J Pathol 200, 423-428.

Boudreau, N., Myers, C., and Bissell, M. J. (1995). From laminin to lamin: regulation of tissue-specific gene expression by the ECM. Trends Cell Biol 5, 1-4.

Brakebusch, C., Bouvard, D., Stanchi, F., Sakai, T., and Fassler, R. (2002). Integrins in invasive growth. J Clin Invest 109, 999-1006.

Brinkerhoff, C. J., and Linderman, J. J. (2005). Integrin dimerization and ligand organization: key components in integrin clustering for cell adhesion. Tissue Eng 11, 865-876.

Carty, M. D., Lillquist, J. S., Peshavaria, M., Stein, R., and Soeller, W. C. (1997). Identification of cis- and trans-active factors regulating human islet amyloid polypeptide gene expression in pancreatic beta cells. J Biol Chem 272, 11986-11993.

Cha J. Y., K. H. Kim K. S, Hur M.W., Ahn Y. (2000). Identification of transacting factors responsible for the tissue-specific expression of human glucose transporter type 2 isoform gene. Cooperative role of hepatocyte nuclear factors 1alpha and 3beta. J Biol Chem 275, 18358-18365.

Chen, F., Zhang, G., Iwamoto, Y., and See, W. A. (2005). BCG directly induces cell cycle arrest in human transitional carcinoma cell lines as a consequence of integrin cross-linking. BMC Urol 5, 8.

Chen, Q., Lin, T. H., Der, C. J., and Juliano, R. L. (1996). Integrin-mediated activation of MEK and mitogen-activated protein kinase is independent of Ras [corrected]. J Biol Chem 271, 18122-18127.

Clark, A., Bown, E., King, T., Vanhegan, R. I., and Turner, R. C. (1982). Islet changes induced by hyperglycemia in rats. Effect of insulin or chlorpropamide therapy. Diabetes 31, 319-325.

Cleaver, O., and Melton, D. A. (2003). Endothelial signaling during development. Nat Med 9, 661-668.

Colognato, H., and Yurchenco, P. D. (2000). Form and function: the laminin family of heterotrimers. Dev Dyn 218, 213-234.

Costell, M., Gustafsson, E., Aszodi, A., Morgelin, M., Bloch, W., Hunziker, E., Addicks, K., Timpl, R., and Fassler, R. (1999). Perlecan maintains the integrity of cartilage and some basement membranes. J Cell Biol 147, 1109-1122.

Cousin, S. P., Hugl, S. R., Myers, M. G., Jr., White, M. F., Reifel-Miller, A., and Rhodes, C. J. (1999). Stimulation of pancreatic beta-cell proliferation by growth hormone is glucose-dependent: signal transduction via Janus kinase 2 (JAK2)/signal transducer and activator of transcription 5 (STAT5) with no crosstalk to insulin receptor substrate-mediated mitogenic signalling. Biochem J 344 Pt 3, 649-658.

Daniel, P. B., Walker, W. H., and Habener, J. F. (1998). Cyclic AMP signaling and gene regulation. Annu Rev Nutr 18, 353-383.

Davies, P. O., Poirier, C., Deltour, L., and Montagutelli, X. (1994). Genetic reassignment of the insulin-1 (Ins1) gene to distal mouse chromosome 19. Genomics 21, 665-667.

Deltour, L., Leduque, P., Blume, N., Madsen, O., Dubois, P., Jami, J., and Bucchini, D. (1993). Differential expression of the two nonallelic proinsulin genes in the developing mouse embryo. Proc Natl Acad Sci U S A 90, 527-531.

Doi, M., Thyboll, J., Kortesmaa, J., Jansson, K., Iivanainen, A., Parvardeh, M., Timpl, R., Hedin, U., Swedenborg, J., and Tryggvason, K. (2002). Recombinant human laminin-10 (alpha5beta1gamma1). Production, purification, and migration-promoting activity on vascular endothelial cells. J Biol Chem 277, 12741-12748.

Dor, Y., Brown, J., Martinez, O. I., and Melton, D. A. (2004). Adult pancreatic beta cells are formed by self-duplication rather than stem cell differentiation. Nature 429, 41-46.

Dukes, I. D., Sreenan, S., Roe, M. W., Levisetti, M., Zhou, Y. P., Ostrega, D., Bell, G. I., Pontoglio, M., Yaniv, M., Philipson, L., and Polonsky, K. S. (1998). Defective pancreatic beta cell glycolytic signaling in hepatocyte nuclear factor-1alpha-deficient mice. J Biol Chem 273, 24457-24464.

Duvillie, B., Bucchini, D., Tang, T., Jami, J., and Paldi, A. (1998). Imprinting at the mouse Ins2 locus: evidence for cis- and trans-allelic interactions. Genomics 47, 52-57.

Duvillie, B., Currie, C., Chrones, T., Bucchini, D., Jami, J., Joshi, R. L., and Hill, D. J. (2002). Increased islet cell proliferation, decreased apoptosis, and greater vascularization leading to beta cell hyperplasia in mutant mice lacking insulin. Endocrinology 143, 1530-1537.

Edlund, H. (2002). Pancreatic organogenesis--developmental mechanisms and implications for therapy. Nat Rev Genet 3, 524-532.

Edvell, A., and Lindstrom, P. (1999). Initiation of increased pancreatic islet growth in young normoglycemic mice (Umea +/?). Endocrinology 140, 778-783.

Finegood, D. T., Scaglia, L., and Bonner-Weir, S. (1995). Dynamics of beta-cell mass in the growing rat pancreas. Estimation with a simple mathematical model. Diabetes 44, 249-256.

Flamez, D., Gilon, P., Moens, K., Van Breusegem, A., Delmeire, D., Scrocchi, L. A., Henquin, J. C., Drucker, D. J., and Schuit, F. (1999). Altered cAMP and Ca2+ signaling in mouse pancreatic islets with glucagon-like peptide-1 receptor null phenotype. Diabetes 48, 1979-1986.

Florey (1966). The endothelial cell. Br Med J 5512, 487-490.

Friedrichsen, B. N., Galsgaard, E. D., Nielsen, J. H., and Moldrup, A. (2001). Growth hormone- and prolactin-induced proliferation of insulinoma cells, INS-1, depends on activation of STAT5 (signal transducer and activator of transcription 5). Mol Endocrinol 15, 136-148.

Gerber, H. P., Hillan, K. J., Ryan, A. M., Kowalski, J., Keller, G. A., Rangell, L., Wright, B. D., Radtke, F., Aguet, M., and Ferrara, N. (1999). VEGF is required for growth and survival in neonatal mice. Development 126, 1149-1159.

German, M., Ashcroft, S., Docherty, K., Edlund, H., Edlund, T., Goodison, S., Imura, H., Kennedy, G., Madsen, O., Melloul, D., and et al. (1995). The insulin gene promoter. A simplified nomenclature. Diabetes 44, 1002-1004.

Giancotti, F. G., and Ruoslahti, E. (1999). Integrin signaling. Science 285, 1028-1032.

Gu, G., Brown, J. R., and Melton, D. A. (2003). Direct lineage tracing reveals the ontogeny of pancreatic cell fates during mouse embryogenesis. Mech Dev 120, 35-43.

Gu, G., Wells, J. M., Dombkowski, D., Preffer, F., Aronow, B., and Melton, D. A. (2004). Global expression analysis of gene regulatory pathways during endocrine pancreatic development. Development 131, 165-179.

Gu, J. (2004). Beyound Glycogenesis. Glycoforum.

Guo, W., and Giancotti, F. G. (2004). Integrin signalling during tumour progression. Nat Rev Mol Cell Biol 5, 816-826.

Guz, Y., Nasir, I., and Teitelman, G. (2001). Regeneration of pancreatic beta cells from intra-islet precursor cells in an experimental model of diabetes. Endocrinology 142, 4956-4968.

Habener, J. F., Kemp, D. M., and Thomas, M. K. (2005). Minireview: transcriptional regulation in pancreatic development. Endocrinology 146, 1025-1034.

Hammar, E. B., Irminger, J. C., Rickenbach, K., Parnaud, G., Ribaux, P., Bosco, D., Rouiller, D. G., and Halban, P. A. (2005). Activation of NF-kappaB by extracellular matrix is involved in spreading and glucose-stimulated insulin secretion of pancreatic beta cells. J Biol Chem 280, 30630-30637.

Hanahan, D., and Folkman, J. (1996). Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 86, 353-364.

Harper, M. E., Ullrich, A., and Saunders, G. F. (1981). Localization of the human insulin gene to the distal end of the short arm of chromosome 11. Proc Natl Acad Sci U S A 78, 4458-4460.

Harrington, R. H., and Sharma, A. (2001). Transcription factors recognizing overlapping C1-A2 binding sites positively regulate insulin gene expression. J Biol Chem 276, 104-113.

Hato, T., Pampori, N., and Shattil, S. J. (1998). Complementary roles for receptor clustering and conformational change in the adhesive and signaling functions of integrin alphaIIb beta3. J Cell Biol 141, 1685-1695.

Hellerstrom, C., Swenne, I., and Eriksson, U. J. (1985). Is there an animal model for gestational diabetes? Diabetes 34 Suppl 2, 28-31.

Henderson, J. R., and Moss, M. C. (1985). A morphometric study of the endocrine and exocrine capillaries of the pancreas. Q J Exp Physiol 70, 347-356.

Henschel, A., Buchholz, F., and Habermann, B. (2004). DEQOR: a web-based tool for the design and quality control of siRNAs. Nucleic Acids Res 32, W113-120.

Herrington, J., Smit, L. S., Schwartz, J., and Carter-Su, C. (2000). The role of STAT proteins in growth hormone signaling. Oncogene 19, 2585-2597.

Hill, C. S., and Treisman, R. (1995). Transcriptional regulation by extracellular signals: mechanisms and specificity. Cell 80, 199-211.

Hill, D. J., and Duvillie, B. (2000). Pancreatic development and adult diabetes. Pediatr Res 48, 269-274.

Hopf, M., Gohring, W., Kohfeldt, E., Yamada, Y., and Timpl, R. (1999). Recombinant domain IV of perlecan binds to nidogens, laminin-nidogen complex, fibronectin, fibulin-2 and heparin. Eur J Biochem 259, 917-925.

Huang, C. C., Hall, D. H., Hedgecock, E. M., Kao, G., Karantza, V., Vogel, B. E., Hutter, H., Chisholm, A. D., Yurchenco, P. D., and Wadsworth, W. G. (2003). Laminin alpha subunits and their role in C. elegans development. Development 130, 3343-3358.

Hudson, B. G., Reeders, S. T., and Tryggvason, K. (1993). Type IV collagen: structure, gene organization, and role in human diseases. Molecular basis of Goodpasture and Alport syndromes and diffuse leiomyomatosis. J Biol Chem 268, 26033-26036.

Humphries, M. J., McEwan, P. A., Barton, S. J., Buckley, P. A., Bella, J., and Mould, A. P. (2003). Integrin structure: heady advances in ligand binding, but activation still makes the knees wobble. Trends Biochem Sci 28, 313-320.

Hutley, L. J., Herington, A. C., Shurety, W., Cheung, C., Vesey, D. A., Cameron, D. P., and Prins, J. B. (2001). Human adipose tissue endothelial cells promote preadipocyte proliferation. Am J Physiol Endocrinol Metab 281, E1037-1044.

Hynes, R. O. (2002). Integrins: bidirectional, allosteric signaling machines. Cell 110, 673-687.

Hynes, R. O., Schwarzbauer, J. E., and Tamkun, J. W. (1987). Isolation and analysis of cDNA and genomic clones of fibronectin and its receptor. Methods Enzymol 144, 447-463.

Ianus, A., Holz, G. G., Theise, N. D., and Hussain, M. A. (2003). In vivo derivation of glucose-competent pancreatic endocrine cells from bone marrow without evidence of cell fusion. J Clin Invest 111, 843-850.

Inoue, M., Hager, J. H., Ferrara, N., Gerber, H. P., and Hanahan, D. (2002). VEGF-A has a critical, nonredundant role in angiogenic switching and pancreatic beta cell carcinogenesis. Cancer Cell 1, 193-202.

Itoh, N., and Okamoto, H. (1980). Translational control of proinsulin synthesis by glucose. Nature 283, 100-102.

Iype, T., Francis, J., Garmey, J. C., Schisler, J. C., Nesher, R., Weir, G. C., Becker, T. C., Newgard, C. B., Griffen, S. C., and Mirmira, R. G. (2005). Mechanism of insulin gene regulation by the pancreatic transcription factor Pdx-1: application of pre-mRNA analysis and chromatin immunoprecipitation to assess formation of functional transcriptional complexes. J Biol Chem 280, 16798-16807.

Jansson, L., and Carlsson, P. O. (2002). Graft vascular function after transplantation of pancreatic islets. Diabetologia 45, 749-763.

Juliano, R. (1996). Cooperation between soluble factors and integrin-mediated cell anchorage in the control of cell growth and differentiation. Bioessays 18, 911-917.

Jun, H. S., and Yoon, J. W. (2005). Approaches for the cure of type 1 diabetes by cellular and gene therapy. Curr Gene Ther 5, 249-262.

Kaido, T., Yebra, M., Cirulli, V., and Montgomery, A. M. (2004). Regulation of human beta-cell adhesion, motility, and insulin secretion by collagen IV and its receptor alpha1beta1. J Biol Chem 279, 53762-53769.

Kaneto, H., Matsuoka, T. A., Nakatani, Y., Miyatsuka, T., Matsuhisa, M., Hori, M., and Yamasaki, Y. (2005). A crucial role of MafA as a novel therapeutic target for diabetes. J Biol Chem 280, 15047-15052.

Kanwar, Y. S., and Farquhar, M. G. (1979). Isolation of glycosaminoglycans (heparan sulfate) from glomerular basement membranes. Proc Natl Acad Sci U S A 76, 4493-4497.

Kataoka, K., Han, S. I., Shioda, S., Hirai, M., Nishizawa, M., and Handa, H. (2002). MafA is a glucose-regulated and pancreatic beta-cell-specific transcriptional activator for the insulin gene. J Biol Chem 277, 49903-49910.

Kataoka, K., Shioda, S., Ando, K., Sakagami, K., Handa, H., and Yasuda, K. (2004). Differentially expressed Maf family transcription factors, c-Maf and MafA, activate glucagon and insulin gene expression in pancreatic islet alpha- and beta-cells. J Mol Endocrinol 32, 9-20.

Kaufman, D. B., and Lowe, W. L., Jr. (2003). Clinical islet transplantation. Curr Diab Rep 3, 344-350.

Kennedy, G. C., and Rutter, W. J. (1992). Pur-1, a zinc-finger protein that binds to purine-rich sequences, transactivates an insulin promoter in heterologous cells. Proc Natl Acad Sci U S A 89, 11498-11502.

Kitamoto, Y., Tokunaga, H., and Tomita, K. (1997). Vascular endothelial growth factor is an essential molecule for mouse kidney development: glomerulogenesis and nephrogenesis. J Clin Invest 99, 2351-2357.

Kittler, R., Putz, G., Pelletier, L., Poser, I., Heninger, A. K., Drechsel, D., Fischer, S., Konstantinova, I., Habermann, B., Grabner, H., et al. (2004). An endoribonuclease-prepared siRNA screen in human cells identifies genes essential for cell division. Nature 432, 1036-1040.

Knoch, K. P., Bergert, H., Borgonovo, B., Saeger, H. D., Altkruger, A., Verkade, P., and Solimena, M. (2004). Polypyrimidine tract-binding protein promotes insulin secretory granule biogenesis. Nat Cell Biol 6, 207-214.

Kodama, S., Kuhtreiber, W., Fujimura, S., Dale, E. A., and Faustman, D. L. (2003). Islet regeneration during the reversal of autoimmune diabetes in NOD mice. Science 302, 1223-1227.

Koranyi, L., Permutt, M. A., Chirgwin, J. M., and Giddings, S. J. (1989). Proinsulin I and II gene expression in inbred mouse strains. Mol Endocrinol 3, 1895-1902.

Kortesmaa, J., Yurchenco, P., and Tryggvason, K. (2000). Recombinant laminin-8 (alpha(4)beta(1)gamma(1)). Production, purification, and interactions with integrins. J Biol Chem 275, 14853-14859.

Lammert, E., Cleaver, O., and Melton, D. (2001). Induction of pancreatic differentiation by signals from blood vessels. Science 294, 564-567.

Lammert, E., Gu, G., McLaughlin, M., Brown, D., Brekken, R., Murtaugh, L. C., Gerber, H. P., Ferrara, N., and Melton, D. A. (2003). Role of VEGF-A in vascularization of pancreatic islets. Curr Biol 13, 1070-1074.

LeCouter, J., Kowalski, J., Foster, J., Hass, P., Zhang, Z., Dillard-Telm, L., Frantz, G., Rangell, L., DeGuzman, L., Keller, G. A., et al. (2001). Identification of an angiogenic mitogen selective for endocrine gland endothelium. Nature 412, 877-884.

LeCouter, J., Moritz, D. R., Li, B., Phillips, G. L., Liang, X. H., Gerber, H. P., Hillan, K. J., and Ferrara, N. (2003). Angiogenesis-independent endothelial protection of liver: role of VEGFR-1. Science 299, 890-893.

Leonard, J., Peers, B., Johnson, T., Ferreri, K., Lee, S., and Montminy, M. R. (1993). Characterization of somatostatin transactivating factor-1, a novel homeobox factor that stimulates somatostatin expression in pancreatic islet cells. Mol Endocrinol 7, 1275-1283.

Lerner, L., and Torchia, D. A. (1986). A multinuclear NMR study of the interactions of cations with proteoglycans, heparin, and Ficoll. J Biol Chem 261, 12706-12714

Desbois, P., Lamotte, L., Duvillie, B., Cordonnier, N., Jackerott, M., Jami, J., Leroux, L., Bucchini, D., and Joshi, R. L. (2001). Compensatory responses in mice carrying a null mutation for Ins1 or Ins2. Diabetes 50 Suppl 1, S150-153.

Letters to the editor (1999). Diabetes research and clinical practice.

Li, M. L., Aggeler, J., Farson, D. A., Hatier, C., Hassell, J., and Bissell, M. J. (1987). Influence of a reconstituted basement membrane and its components on casein gene expression and secretion in mouse mammary epithelial cells. Proc Natl Acad Sci U S A 84, 136-140.

Li, S., Harrison, D., Carbonetto, S., Fassler, R., Smyth, N., Edgar, D., and Yurchenco, P. D. (2002). Matrix assembly, regulation, and survival functions of laminin and its receptors in embryonic stem cell differentiation. J Cell Biol 157, 1279-1290.

Liddington, R. C. (2002). Will the real integrin please stand up? Structure 10, 605-607.

Liddington, R. C., and Ginsberg, M. H. (2002). Integrin activation takes shape. J Cell Biol 158, 833-839.

Lin, T. H., Aplin, A. E., Shen, Y., Chen, Q., Schaller, M., Romer, L., Aukhil, I., and Juliano, R. L. (1997). Integrin-mediated activation of MAP kinase is independent of FAK: evidence for dual integrin signaling pathways in fibroblasts. J Cell Biol 136, 1385-1395.

Linde, S., Nielsen, J. H., Hansen, B., and Welinder, B. S. (1989). Reversed-phase high-performance liquid chromatographic analyses of insulin biosynthesis in isolated rat and mouse islets. J Chromatogr 462, 243-254.

Lipsett, M., and Finegood, D. T. (2002). beta-cell neogenesis during prolonged hyperglycemia in rats. Diabetes 51, 1834-1841.

Liu, J., DeYoung, S. M., Zhang, M., Zhang, M., Cheng, A., and Saltiel, A. R. (2005). Changes in integrin expression during adipocyte differentiation. Cell Metab 2, 165-177.

Liu, Y. Q., Nevin, P. W., and Leahy, J. L. (2000). beta-cell adaptation in 60% pancreatectomy rats that preserves normoinsulinemia and normoglycemia. Am J Physiol Endocrinol Metab 279, E68-73.

Lohr, M., Lubbersmeyer, J., Otremba, B., Klapdor, R., Grossner, D., and Kloppel, G. (1989). Increase in B-cells in the pancreatic remnant after partial pancreatectomy in pigs. An immunocytochemical and functional study. Virchows Arch B Cell Pathol Incl Mol Pathol 56, 277-286.

Louissaint, A., Jr., Rao, S., Leventhal, C., and Goldman, S. A. (2002). Coordinated interaction of neurogenesis and angiogenesis in the adult songbird brain. Neuron 34, 945-960.

Lucocq, J. (1993). Unbiased 3-D quantitation of ultrastructure in cell biology. Trends Cell Biol 3, 354-358.

Lukinius, A., Jansson, L., and Korsgren, O. (1995). Ultrastructural evidence for blood microvessels devoid of an endothelial cell lining in transplanted pancreatic islets. Am J Pathol 146, 429-435.

Majumdar, A., and Drummond, I. A. (1999). Podocyte differentiation in the absence of endothelial cells as revealed in the zebrafish avascular mutant, cloche. Dev Genet 24, 220-229.

Markussen, J., Heding, L. G., Jorgensen, K. H., and Sundby, F. (1971). Proinsulin, insulin and C-peptide. Horm Metab Res 3, Suppl 3:33-35.

Marshak, S., Benshushan, E., Shoshkes, M., Leibovitz, G., Kaiser, N., Gross, D., Bertuzzi, F., Cerasi, E., and Melloul, D. (2001). beta-cell-specific expression of insulin and PDX-1 genes. Diabetes 50 Suppl 1, S131-132.

Marshak, S., Totary, H., Cerasi, E., and Melloul, D. (1996). Purification of the beta-cell glucose-sensitive factor that transactivates the insulin gene differentially in normal and transformed islet cells. Proc Natl Acad Sci U S A 93, 15057-15062.

Martin, D., Zusman, S., Li, X., Williams, E. L., Khare, N., DaRocha, S., Chiquet-Ehrismann, R., and Baumgartner, S. (1999). wing blister, a new Drosophila laminin alpha chain required for cell adhesion and migration during embryonic and imaginal development. J Cell Biol 145, 191-201.

Marynissen, G., Aerts, L., and Van Assche, F. A. (1983). The endocrine pancreas during pregnancy and lactation in the rat. J Dev Physiol 5, 373-381.

Massari, M. E., and Murre, C. (2000). Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms. Mol Cell Biol 20, 429-440.

Matsumoto, K., Yoshitomi, H., Rossant, J., and Zaret, K. S. (2001). Liver organogenesis promoted by endothelial cells prior to vascular function. Science 294, 559-563.

Matsuoka, T. A., Artner, I., Henderson, E., Means, A., Sander, M., and Stein, R. (2004). The MafA transcription factor appears to be responsible for tissue-specific expression of insulin. Proc Natl Acad Sci U S A 101, 2930-2933.

Melloul, D., Marshak, S., and Cerasi, E. (2002). Regulation of insulin gene transcription. Diabetologia 45, 309-326.

Mi, H., Haeberle, H., and Barres, B. A. (2001). Induction of astrocyte differentiation by endothelial cells. J Neurosci 21, 1538-1547.

Miettinen, A., Stow, J. L., Mentone, S., and Farquhar, M. G. (1986). Antibodies to basement membrane heparan sulfate proteoglycans bind to the laminae rarae of the glomerular basement membrane (GBM) and induce subepithelial GBM thickening. J Exp Med 163, 1064-1084.

Miner, J. H. (1998). Developmental biology of glomerular basement membrane components. Curr Opin Nephrol Hypertens 7, 13-19.

Miner, J. H., and Li, C. (2000). Defective glomerulogenesis in the absence of laminin alpha5 demonstrates a developmental role for the kidney glomerular basement membrane. Dev Biol 217, 278-289.

Miner, J. H., Li, C., Mudd, J. L., Go, G., and Sutherland, A. E. (2004). Compositional and structural requirements for laminin and basement membranes during mouse embryo implantation and gastrulation. Development 131, 2247-2256.

Miner, J. H., Patton, B. L., Lentz, S. I., Gilbert, D. J., Snider, W. D., Jenkins, N. A., Copeland, N. G., and Sanes, J. R. (1997). The laminin alpha chains: expression, developmental transitions, and chromosomal locations of alpha1-5, identification of heterotrimeric laminins 8-11, and cloning of a novel alpha3 isoform. J Cell Biol 137, 685-701.

Miyazaki, J., Araki, K., Yamato, E., Ikegami, H., Asano, T., Shibasaki, Y., Oka, Y., and Yamamura, K. (1990). Establishment of a pancreatic beta cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms. Endocrinology 127, 126-132.

Moro, L., Dolce, L., Cabodi, S., Bergatto, E., Erba, E. B., Smeriglio, M., Turco, E., Retta, S. F., Giuffrida, M. G., Venturino, M., et al. (2002). Integrin-induced epidermal growth factor (EGF) receptor activation requires c-Src and p130Cas and leads to phosphorylation of specific EGF receptor tyrosines. J Biol Chem 277, 9405-9414.

Morrison, H. (1937). Contributions to the microscopic anatomy of the pancreas by Paul Langerhans (Berlin, 1869). Bulletin of the Institute of the History of the Medicine 5, 259-297.

Nakajima, Y., Yamagishi, T., Hokari, S., and Nakamura, H. (2000). Mechanisms involved in valvuloseptal endocardial cushion formation in early cardiogenesis: roles of transforming growth factor (TGF)-beta and bone morphogenetic protein (BMP). Anat Rec 258, 119-127.

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

Nielsen, J. H. (1982). Effects of growth hormone, prolactin, and placental lactogen on insulin content and release, and deoxyribonucleic acid synthesis in cultured pancreatic islets. Endocrinology 110, 600-606.

Nielsen, J. H., Galsgaard, E. D., Moldrup, A., Friedrichsen, B. N., Billestrup, N., Hansen, J. A., Lee, Y. C., and Carlsson, C. (2001). Regulation of beta-cell mass by hormones and growth factors. Diabetes 50 Suppl 1, S25-29.

Nielsen, J. H., Nielsen, V., Molsted Pedersen, L., and Deckert, T. (1986). Effects of pregnancy hormones on pancreatic islets in organ culture. Acta Endocrinol (Copenh) 111, 336-341.

Nielsen, K., Karlsen, A. E., Deckert, M., Madsen, O. D., Serup, P., Mandrup-Poulsen, T., and Nerup, J. (1999). Beta-cell maturation leads to in vitro sensitivity to cytotoxins. Diabetes 48, 2324-2332.

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

Ohlsson, H., Karlsson, K., and Edlund, T. (1993). IPF1, a homeodomain-containing transactivator of the insulin gene. Embo J 12, 4251-4259.

Palmer, T. D., Willhoite, A. R., and Gage, F. H. (2000). Vascular niche for adult hippocampal neurogenesis. J Comp Neurol 425, 479-494.

Parsons, J. A., Bartke, A., and Sorenson, R. L. (1995). Number and size of islets of Langerhans in pregnant, human growth hormone-expressing transgenic, and pituitary dwarf mice: effect of lactogenic hormones. Endocrinology 136, 2013-2021.

Parsons, J. A., Brelje, T. C., and Sorenson, R. L. (1992). Adaptation of islets of Langerhans to pregnancy: increased islet cell proliferation and insulin secretion correlates with the onset of placental lactogen secretion. Endocrinology 130, 1459-1466.

Permutt, M. A., and Kipnis, D. M. (1972). Insulin biosynthesis. II. Effect of glucose on ribonucleic acid synthesis in isolated rat islets. J Biol Chem 247, 1200-1207.

Piccardoni, P., Manarini, S., Federico, L., Bagoly, Z., Pecce, R., Martelli, N., Piccoli, A., Totani, L., Cerletti, C., and Evangelista, V. (2004). SRC-dependent outside-in signalling is a key step in the process of autoregulation of beta2 integrins in polymorphonuclear cells. Biochem J 380, 57-65.

Poschl, E., Schlotzer-Schrehardt, U., Brachvogel, B., Saito, K., Ninomiya, Y., and Mayer, U. (2004). Collagen IV is essential for basement membrane stability but dispensable for initiation of its assembly during early development. Development 131, 1619-1628.

Reinhardt, D., Mann, K., Nischt, R., Fox, J. W., Chu, M. L., Krieg, T., and Timpl, R. (1993). Mapping of nidogen binding sites for collagen type IV, heparan sulfate proteoglycan, and zinc. J Biol Chem 268, 10881-10887.

Roderigo-Milne, H., Hauge-Evans, A. C., Persaud, S. J., and Jones, P. M. (2002). Differential expression of insulin genes 1 and 2 in MIN6 cells and pseudoislets. Biochem Biophys Res Commun 296, 589-595.

Ruoslahti, E. (2002). Specialization of tumour vasculature. Nat Rev Cancer 2, 83-90.

Ruoslahti, E., and Pierschbacher, M. D. (1987). New perspectives in cell adhesion: RGD and integrins. Science 238, 491-497.

Sandler, S., and Andersson, A. (1986). Long-term effects of exposure of pancreatic islets to nicotinamide in vitro on DNA synthesis, metabolism and B-cell function. Diabetologia 29, 199-202.

Scaglia, L., Smith, F. E., and Bonner-Weir, S. (1995). Apoptosis contributes to the involution of beta cell mass in the post partum rat pancreas. Endocrinology 136, 5461-5468.

Schlaepfer, D. D., Hanks, S. K., Hunter, T., and van der Geer, P. (1994). Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. Nature 372, 786-791.

Schmidhauser, C., Casperson, G. F., Myers, C. A., Sanzo, K. T., Bolten, S., and Bissell, M. J. (1992). A novel transcriptional enhancer is involved in the prolactin- and extracellular matrix-dependent regulation of beta-casein gene expression. Mol Biol Cell 3, 699-709.

Schwartz, M. A., and Baron, V. (1999). Interactions between mitogenic stimuli, or, a thousand and one connections. Curr Opin Cell Biol 11, 197-202.

Schwarzbauer, J. (1999). Basement membranes: Putting up the barriers. Curr Biol 9, R242-244.

Sebzda, E., Bracke, M., Tugal, T., Hogg, N., and Cantrell, D. A. (2002). Rap1A positively regulates T cells via integrin activation rather than inhibiting lymphocyte signaling. Nat Immunol 3, 251-258

Seger, R., and Krebs, E. G. (1995). The MAPK signaling cascade. Faseb J 9, 726-735.

Seid, C. A., Ramachandran, R. K., George, J. M., Govindarajan, V., Gonzalez-Rimbau, M. F., Flytzanis, C. N., and Tomlinson, C. R. (1997). An extracellular matrix response element in the promoter of the LpS1 genes of the sea urchin Lytechinus pictus. Nucleic Acids Res 25, 3175-3182.

Sekine, N., Wollheim, C. B., and Fujita, T. (1998). GH signalling in pancreatic beta-cells. Endocr J 45 Suppl, S33-40.

Shapiro, R., Randhawa, P., Jordan, M. L., Scantlebury, V. P., Vivas, C., Jain, A., Corry, R. J., McCauley, J., Johnston, J., Donaldson, J., et al. (2001). An analysis of early renal transplant protocol biopsies--the high incidence of subclinical tubulitis. Am J Transplant 1, 47-50.

Sharma, A., and Stein, R. (1994). Glucose-induced transcription of the insulin gene is mediated by factors required for beta-cell-type-specific expression. Mol Cell Biol 14, 871-879.

Sharma, S., Jhala, U. S., Johnson, T., Ferreri, K., Leonard, J., and Montminy, M. (1997). Hormonal regulation of an islet-specific enhancer in the pancreatic homeobox gene STF-1. Mol Cell Biol 17, 2598-2604.

Shen, Q., Goderie, S. K., Jin, L., Karanth, N., Sun, Y., Abramova, N., Vincent, P., Pumiglia, K., and Temple, S. (2004). Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells. Science 304, 1338-1340.

Shieh, S. Y., and Tsai, M. J. (1991). Cell-specific and ubiquitous factors are responsible for the enhancer activity of the rat insulin II gene. J Biol Chem 266, 16708-16714.

Simionescu, M., Simionescu, N., and Palade, G. E. (1984). Partial chemical characterization of the anionic sites in the basal lamina of fenestrated capillaries. Microvasc Res 28, 352-367.

Sims, P. J., Ginsberg, M. H., Plow, E. F., and Shattil, S. J. (1991). Effect of platelet activation on the conformation of the plasma membrane glycoprotein IIb-IIIa complex. J Biol Chem 266, 7345-7352.

Sipkins, D. A., Wei, X., Wu, J. W., Runnels, J. M., Cote, D., Means, T. K., Luster, A. D., Scadden, D. T., and Lin, C. P. (2005). In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment. Nature 435, 969-973.

Sjoholm, A. (1991). Phorbol ester stimulation of pancreatic beta-cell replication, polyamine content and insulin secretion. FEBS Lett 294, 257-260.

Sjoholm, A., Welsh, N., and Hellerstrom, C. (1992). Lithium increases DNA replication, polyamine content, and insulin secretion by rat pancreatic beta-cells. Am J Physiol 262, C391-395.

Smyth, N., Vatansever, H. S., Murray, P., Meyer, M., Frie, C., Paulsson, M., and Edgar, D. (1999). Absence of basement membranes after targeting the LAMC1 gene results in embryonic lethality due to failure of endoderm differentiation. J Cell Biol 144, 151-160.

Soares, M. B., Schon, E., Henderson, A., Karathanasis, S. K., Cate, R., Zeitlin, S., Chirgwin, J., and Efstratiadis, A. (1985). RNA-mediated gene duplication: the rat preproinsulin I gene is a functional retroposon. Mol Cell Biol 5, 2090-2103.

Steck, A. K., Bugawan, T. L., Valdes, A. M., Emery, L. M., Blair, A., Norris, J. M., Redondo, M. J., Babu, S. R., Erlich, H. A., Eisenbarth, G. S., and Rewers, M. J. (2005). Association of non-HLA genes with type 1 diabetes autoimmunity. Diabetes 54, 2482-2486.

Steiner, D. F., Clark, J. L., Nolan, C., Rubenstein, A. H., Margoliash, E., Aten, B., and Oyer, P. E. (1969). Proinsulin and the biosynthesis of insulin. Recent Prog Horm Res 25, 207-282.

Stewart, P. A., and Wiley, M. J. (1981). Developing nervous tissue induces formation of blood-brain barrier characteristics in invading endothelial cells: a study using quail--chick transplantation chimeras. Dev Biol 84, 183-192.

Sutherland, D. E., Gruessner, R., Kandswamy, R., Humar, A., Hering, B., and Gruessner, A. (2004). Beta-cell replacement therapy (pancreas and islet transplantation) for treatment of diabetes mellitus: an integrated approach. Transplant Proc 36, 1697-1699.

Svenstrup, K., Skau, M., Pakkenberg, B., Buschard, K., and Bock, T. (2002). Postnatal development of beta-cells in rats. Proposed explanatory model. Apmis 110, 372-378.

Swenne, I. (1992). Pancreatic beta-cell growth and diabetes mellitus. Diabetologia 35, 193-201.

Swenne, I., and Andersson, A. (1984). Effect of genetic background on the capacity for islet cell replication in mice. Diabetologia 27, 464-467.

Swenne, I., Bone, A. J., Howell, S. L., and Hellerstrom, C. (1980). Effects of glucose and amino acids on the biosynthesis of DNA and insulin in fetal rat islets maintained in tissue culture. Diabetes 29, 686-692.

Tadokoro, S., Shattil, S. J., Eto, K., Tai, V., Liddington, R. C., de Pereda, J. M., Ginsberg, M. H., and Calderwood, D. A. (2003). Talin binding to integrin beta tails: a final common step in integrin activation. Science 302, 103-106.

Thyboll, J., Kortesmaa, J., Cao, R., Soininen, R., Wang, L., Iivanainen, A., Sorokin, L., Risling, M., Cao, Y., and Tryggvason, K. (2002). Deletion of the laminin alpha4 chain leads to impaired microvessel maturation. Mol Cell Biol 22, 1194-1202.

Timpl, R., and Brown, J. C. (1996). Supramolecular assembly of basement membranes. Bioessays 18, 123-132.

Timpl, R., Rohde, H., Robey, P. G., Rennard, S. I., Foidart, J. M., and Martin, G. R. (1979). Laminin--a glycoprotein from basement membranes. J Biol Chem 254, 9933-9937.

Tourrel, C., Bailbe, D., Lacorne, M., Meile, M. J., Kergoat, M., and Portha, B. (2002). Persistent improvement of type 2 diabetes in the Goto-Kakizaki rat model by expansion of the beta-cell mass during the prediabetic period with glucagon-like peptide-1 or exendin-4. Diabetes 51, 1443-1452.

Tourrel, C., Bailbe, D., Meile, M. J., Kergoat, M., and Portha, B. (2001). Glucagon-like peptide-1 and exendin-4 stimulate beta-cell neogenesis in streptozotocin-treated newborn rats resulting in persistently improved glucose homeostasis at adult age. Diabetes 50, 1562-1570.

Trajkovski, M. (2005). Regulated secretion and gene expression in beta cells: link through ICA512. Dissertation.

Varzaneh, F. E., Shillabeer, G., Wong, K. L., and Lau, D. C. (1994). Extracellular matrix components secreted by microvascular endothelial cells stimulate preadipocyte differentiation in vitro. Metabolism 43, 906-912.

Villa-Komaroff, L., Efstratiadis, A., Broome, S., Lomedico, P., Tizard, R., Naber, S. P., Chick, W. L., and Gilbert, W. (1992). A bacterial clone synthesizing proinsulin. 1978. Biotechnology 24, 316-320.

Vracko, R. (1974). Basal lamina scaffold-anatomy and significance for maintenance of orderly tissue structure. Am J Pathol 77, 314-346.

Waeber, G., Thompson, N., Nicod, P., and Bonny, C. (1996). Transcriptional activation of the GLUT2 gene by the IPF-1/STF-1/IDX-1 homeobox factor. Mol Endocrinol 10, 1327-1334.

Wang, H., Maechler, P., Hagenfeldt, K. A., and Wollheim, C. B. (1998). Dominant-negative suppression of HNF-1alpha function results in defective insulin gene transcription and impaired metabolism-secretion coupling in a pancreatic beta-cell line. Embo J 17, 6701-6713.

Wang, J. F., Zhang, X. F., and Groopman, J. E. (2001). Stimulation of beta 1 integrin induces tyrosine phosphorylation of vascular endothelial growth factor receptor-3 and modulates cell migration. J Biol Chem 276, 41950-41957.

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

Watada, H., Kajimoto, Y., Umayahara, Y., Matsuoka, T., Kaneto, H., Fujitani, Y., Kamada, T., Kawamori, R., and Yamasaki, Y. (1996b). The human glucokinase gene beta-cell-type promoter: an essential role of insulin promoter factor 1/PDX-1 in its activation in HIT-T15 cells. Diabetes 45, 1478-1488.

Wei, J., Shaw, L. M., and Mercurio, A. M. (1998). Regulation of mitogen-activated protein kinase activation by the cytoplasmic domain of the alpha6 integrin subunit. J Biol Chem 273, 5903-5907.

Welsh, M., Scherberg, N., Gilmore, R., and Steiner, D. F. (1986a). Translational control of insulin biosynthesis. Evidence for regulation of elongation, initiation and signal-recognition-particle-mediated translational arrest by glucose. Biochem J 235, 459-467.

Welsh, N., Sandler, S., Welsh, M., and Hellerstrom, C. (1986b). Regulation of RNA metabolism in relation to insulin production and oxidative metabolism in mouse pancreatic islets in vitro. Biochim Biophys Acta 887, 58-68.

Wennerberg, K., Lohikangas, L., Gullberg, D., Pfaff, M., Johansson, S., and Fassler, R. (1996). Beta 1 integrin-dependent and -independent polymerization of fibronectin. J Cell Biol 132, 227-238.

Wentworth, B. M., Schaefer, I. M., Villa-Komaroff, L., and Chirgwin, J. M. (1986). Characterization of the two nonallelic genes encoding mouse preproinsulin. J Mol Evol 23, 305-312.

Wilson, M. E., Scheel, D., and German, M. S. (2003). Gene expression cascades in pancreatic development. Mech Dev 120, 65-80.

Woodside, D. G., Liu, S., and Ginsberg, M. H. (2001). Integrin activation. Thromb Haemost 86, 316-323.

Wurmser, A. E., Nakashima, K., Summers, R. G., Toni, N., D'Amour, K. A., Lie, D. C., and Gage, F. H. (2004a). Cell fusion-independent differentiation of neural stem cells to the endothelial lineage. Nature 430, 350-356.

Wurmser, A. E., Palmer, T. D., and Gage, F. H. (2004b). Neuroscience. Cellular interactions in the stem cell niche. Science 304, 1253-1255.

Xiong, J. P., Stehle, T., Goodman, S. L., and Arnaout, M. A. (2003). Integrins, cations and ligands: making the connection. J Thromb Haemost 1, 1642-1654.

Yamada, K. M. and Danen, E. H. J. Integrin signaling. In Signalling Networks and Cell Cycle Control (Humana Press) 1-25.

Yokosaki, Y., Monis, H., Chen, J., and Sheppard, D. (1996). Differential effects of the integrins alpha9beta1, alphavbeta3, and alphavbeta6 on cell proliferative responses to tenascin. Roles of the beta subunit extracellular and cytoplasmic domains. J Biol Chem 271, 24144-24150.

Yoshitomi, H., and Zaret, K. S. (2004). Endothelial cell interactions initiate dorsal pancreas development by selectively inducing the transcription factor Ptf1a. Development 131, 807-817.

Yurchenco, P. D., and O'Rear, J. J. (1994). Basement membrane assembly. Methods Enzymol 245, 489-518.

Yurchenco, P. D., Quan, Y., Colognato, H., Mathus, T., Harrison, D., Yamada, Y., and O'Rear, J. J. (1997). The alpha chain of laminin-1 is independently secreted and drives secretion of its beta- and gamma-chain partners. Proc Natl Acad Sci U S A 94, 10189-10194.

Yurchenco, P. D., Smirnov, S., and Mathus, T. (2002). Analysis of basement membrane self-assembly and cellular interactions with native and recombinant glycoproteins. Methods Cell Biol 69, 111-144.

Yurchenco, P. D., and Wadsworth, W. G. (2004). Assembly and tissue functions of early embryonic laminins and netrins. Curr Opin Cell Biol 16, 572-579.

Zajicek, G., Arber, N., Schwartz-Arad, D., and Ariel, I. (1990). Streaming pancreas: islet cell kinetics. Diabetes Res 13, 121-125.

Zaret, K. (2004). Regenerative medicine: self-help for insulin cells. Nature 429, 30-31.

Zhang, N., Richter, A., Suriawinata, J., Harbaran, S., Altomonte, J., Cong, L., Zhang, H., Song, K., Meseck, M., Bromberg, J., and Dong, H. (2004). Elevated vascular endothelial growth factor production in islets improves islet graft vascularization. Diabetes 53, 963-970.

Zulewski, H., Abraham, E. J., Gerlach, M. J., Daniel, P. B., Moritz, W., Muller, B., Vallejo, M., Thomas, M. K., and Habener, J. F. (2001). Multipotential nestin-positive stem cells isolated from adult pancreatic islets differentiate ex vivo into pancreatic endocrine, exocrine, and hepatic phenotypes. Diabetes 50, 521-533

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I herewith declare that I have produced this paper without the prohibited assistance of third parties and without making use of aids other than those specified; notions taken over directly or indirectly from other sources have been identified as such. This paper has not previously been presented in identical or similar form to any other German or foreign examination board.

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I declare that I have not undertaken any previous unsuccessful doctorate proceedings.

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