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The Vascular Basement Membrane: A Niche for Insulin Gene Expression and β Cell Proliferation

Short Article

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Summary

Endocrine pancreatic β cells require endothelial signals for their differentiation and function. However, the molecular basis for such signals remains unknown. Here, we show 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 the β cells. We have identified laminins, among other vascular basement membrane proteins, as endothelial signals, which promote insulin gene expression and proliferation in β cells. We 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 apply to other cellular processes in which endothelial signals are required.

Introduction

Pancreatic islets, and endocrine glands in general, intimately interact with endothelial cells. In the embryo, differentiation and delamination of insulin-producing β cells from pancreatic epithelium strictly require endothelial cells (Lammert et al., 2001; Yoshitomi and Zaret, 2004). During later embryonic development, delaminated β cells aggregate to form islets. These cell aggregates express high levels of the vascular endothelial growth factor VEGF-A (Inoue et al., 2002; Lammert et al., 2003) to attract VEGFR2-expressing endothelial cells, which form a vascular network within the islets. We have recently shown that this particularly dense vascular network is required for proper endocrine function and islet size (Lammert et al., 2003). The expression of an endocrine gland-specific VEGF (EG-VEGF) in some endocrine tissues further highlights the special interaction of endocrine cells with endothelial cells (LeCouter et al., 2001).

From a medical point of view, islet transplantation is one of the most promising approaches to cure diabetes mellitus (Couzin, 2004), but it also has to take endothelial cells into consideration. The enzymatic digestion procedure, used to isolate islets, partially removes intraislet endothelial cells and thereby contributes to transplant failure (Konstantinova and Lammert, 2004; Lukinius et al., 1995). These findings raise the question of whether endothelial cells create a permissive environment, or a vascular niche (Palmer et al., 2000), for β cell function and growth. Vascular niches also play a role in various other processes, including liver organogenesis and growth (LeCouter et al., 2003; Matsumoto et al., 2001), kidney morphogenesis (Serluca et al., 2002), 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). Identification of the molecular mechanisms involved in the interaction between the β cells and endothelial cells would therefore have a general impact on our understanding of tissue function, growth, and disease (Cleaver and Melton, 2003; Nikolova and Lammert, 2003).

Results

Basement Membrane Formation within Pancreatic Islets Requires Vascular Endothelial Cells

In an attempt to identify endothelial signals involved in endocrine function and islet growth, we considered the vascular basement membrane as a possible source of such signals. We stained mouse pancreas sections with antisera raised against laminin and collagen IV, the major protein components of all basement membranes (Hallmann et al., 2005). Figure 1 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 1A and 1C). Previous differential expression analyses have shown that endocrine pancreatic precursor cells and islets downregulate gene expression of many basement membrane proteins (Gu et al., 2004). To investigate whether β cells require endothelial cells for synthesizing the intraislet basement membrane, we used mice with a VEGF-A deletion in pancreatic epithelial cells (Pdx1-Cre × VEGFloxP) (Lammert et al.,

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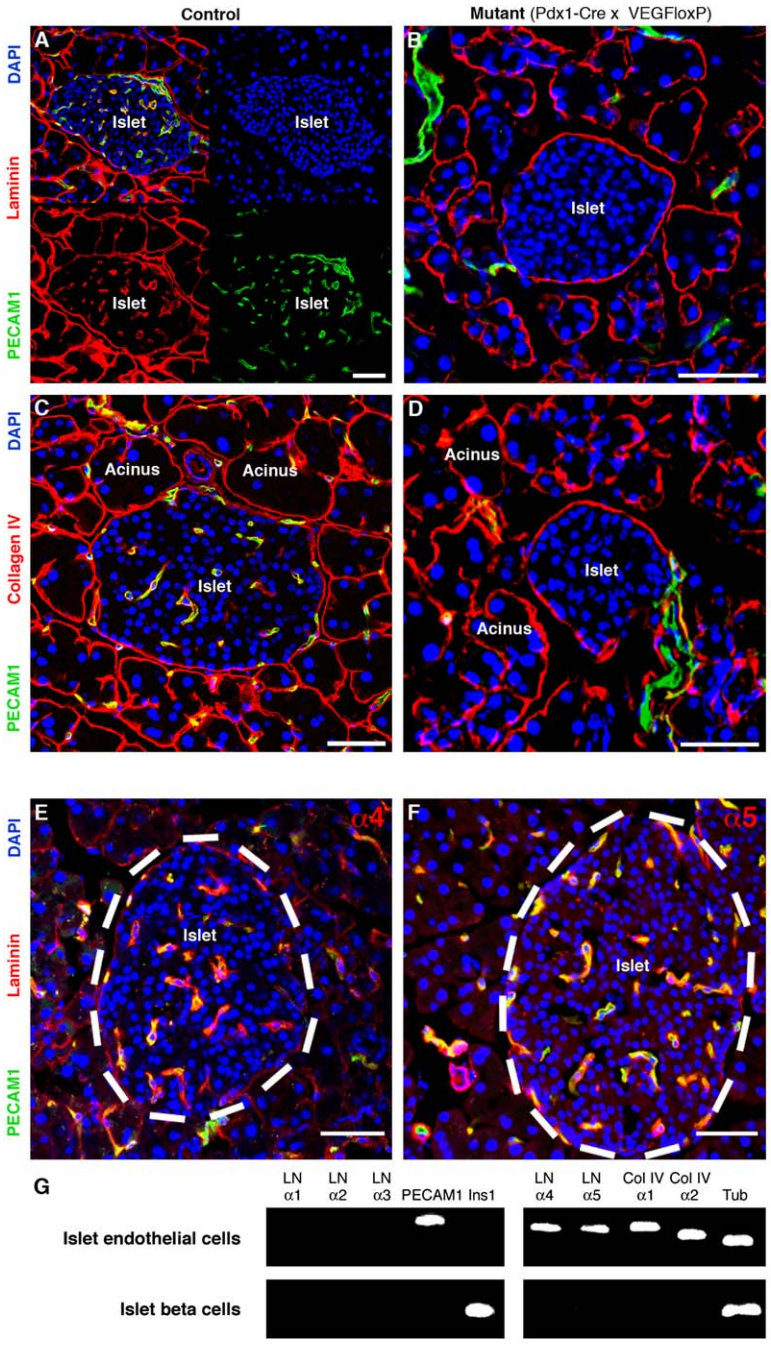

Figure 1. Basement Membrane Formation within Pancreatic Islets Requires Vascular Endothelial Cells

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

(E and F) 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 (E) α4 and (F) α5. The scale bars are 50 μm.

(G) Reverse transcription-polymerase chain reaction with mRNA isolated from mouse islet endothelial cells and β 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.

2003) (Figures 1B and 1D). 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). We showed that the loss of endothelial cells resulted in a loss of the intraislet basement membrane (compare Figure 1B with Figure 1A and Figure 1D with Figure 1C). However, the reduced number of endothelial cells affected neither the basement membrane of the exocrine acini nor the matrix of the islet capsule (Figures 1B and 1D). Thus, in contrast to acinar cells, β cells are unable to form a basement membrane on their own, and they therefore require endothelial cells.

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), we stained mouse pancreas sections for the laminin α4 and α5 chains. As shown in Figures 1E and 1F, 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).

Next, we looked at the gene expression of these laminin chains in endothelial cells and β cells that were sorted from pancreatic islets by FACS (Figure 1G). 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 β cells revealed no major crosscontamination between the sorted cell populations (Figure 1G). Importantly, β cells did not express any laminin α chains, while endothelial cells expressed laminin chains α4 and α5 (Figure 1G) and, occasionally, α1 (data not shown). 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), we conclude that β cells cannot form a basement membrane. Additionally, islet endothelial cells, but not β cells, expressed collagen IV chains α1 and α2 (Figure 1G). These results demonstrate that β cells use VEGF-A to attract endothelial cells, so these cells can form a basement membrane adjacent to the β cells.

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. We found that β 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 2A–2D). In contrast, numbers of other organelles (Figure 2C), as well as expression of control genes (Figures 2E and 2F), were not significantly changed.

We then performed in vitro experiments to test if the vascular basement membrane contained signals capable of regulating insulin gene expression in β cells (Figures 2G–2J). For these experiments, we used mouse insulinoma cells (MIN6). We observed increased insulin gene expression when cells were plated on a reconstituted basement membrane, growth factor-deprived matrigel (compare MG with UT in Figure 2G). 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 2G), 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 2H). Among the proteins tested, laminin-111 upregulated insulin gene expression most strongly (Figure 2H). However, when this laminin in soluble form was provided to the media of the cells, higher concentrations had to be used, and insulin gene expression was increased to a lesser extent (Figure 2I), suggesting that β cells needed to interact with a critical density of laminin molecules in order to activate insulin gene expression.

Most experiments described in this study used laminin-111 (also called EHS-laminin or laminin-1), which was available in amounts sufficient for the experiments described here, as the prototype of laminins. To investigate whether the upregulation of insulin gene expression in β 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 we identified in the islet endothelial cells (Figures 1E–1G). The experiments demonstrated that all laminins upregulated insulin gene expression, but that they did not affect the expression of two control genes, β2-microglobulin and cyclophilin (Figures 2J–2L).

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 2M). Moreover, laminin-111, as well as the vascular laminins (laminin-411 and -511), was able to upregulate insulin gene expression in mutant islets (Figure 2N). Similar to MIN6 cells, the effect of these laminins was inhibited by using anti-β1-integrin blocking antibodies (Figures 2J and 2N). Insulin gene expression could also be increased in control islets, but to a lesser extent than in mutant islets (Figure 2M). We conclude that laminins, among other proteins of the vascular basement membrane, act as endothelial signals, which increase insulin gene expression in β cells.

β1-Integrin Is Required for Upregulating Insulin Gene Expression in Response to Laminins

Next, we investigated whether the β1-integrin subunit, which forms laminin receptors with various α-integrin subunits, was required for the β 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 3A). We also found that the β1-integrin was localized on the plasma membranes of both MIN6 cells (Figure 3B) and pancreatic islet cells (Figure 3D). High-pressure perfusion of mice was then applied to separate the endothelial cells from β cells and show that the β1-integrin was localized on the β cell plasma membrane, which faced the endothelial cell-derived basement membrane (Figure 3E). Thus, based on its expression and localization, β1-integrin on β cells could directly interact with the vascular basement membrane.

We used two strategies to investigate if this integrin was required for responding to laminins. First, we 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 β cell response to laminins (Figures 2J and 2N). Second, we decreased the β1-integrin mRNA level by 80%–90% by using two sets of siRNA molecules (Figures 3G and 3K). This resulted in a 60%–80% reduced β1-integrin cell surface expression (Figures 3I and 3L). With this approach, we found that β1-integrin was strictly required for upregulating insulin gene expression in response to laminin-111 (Figures 3J and 3M). In contrast, it was not required for insulin gene expression in untreated cells (compare UT with LN-111 in Figures 3J and 3M).

Because of the possible role of the α6β1-integrin in insulin secretion (Bosco et al., 2000), we also studied the expression and localization of the α6-integrin. We found that α6-integrin was localized on the plasma membranes of MIN6 cells (Figure 3C) and islet β cells (Figure 3F), similar to the localization of β1-integrin (Figures 3B, 3D, and 3E). Knockdown of α6-integrin by using two sets of siRNA molecules decreased the response of MIN6 cells to laminin-111 (Figures 3Q and 3T). However, in contrast to the β1-integrin knockdown, α6-integrin knockdown only partially impaired this response (compare

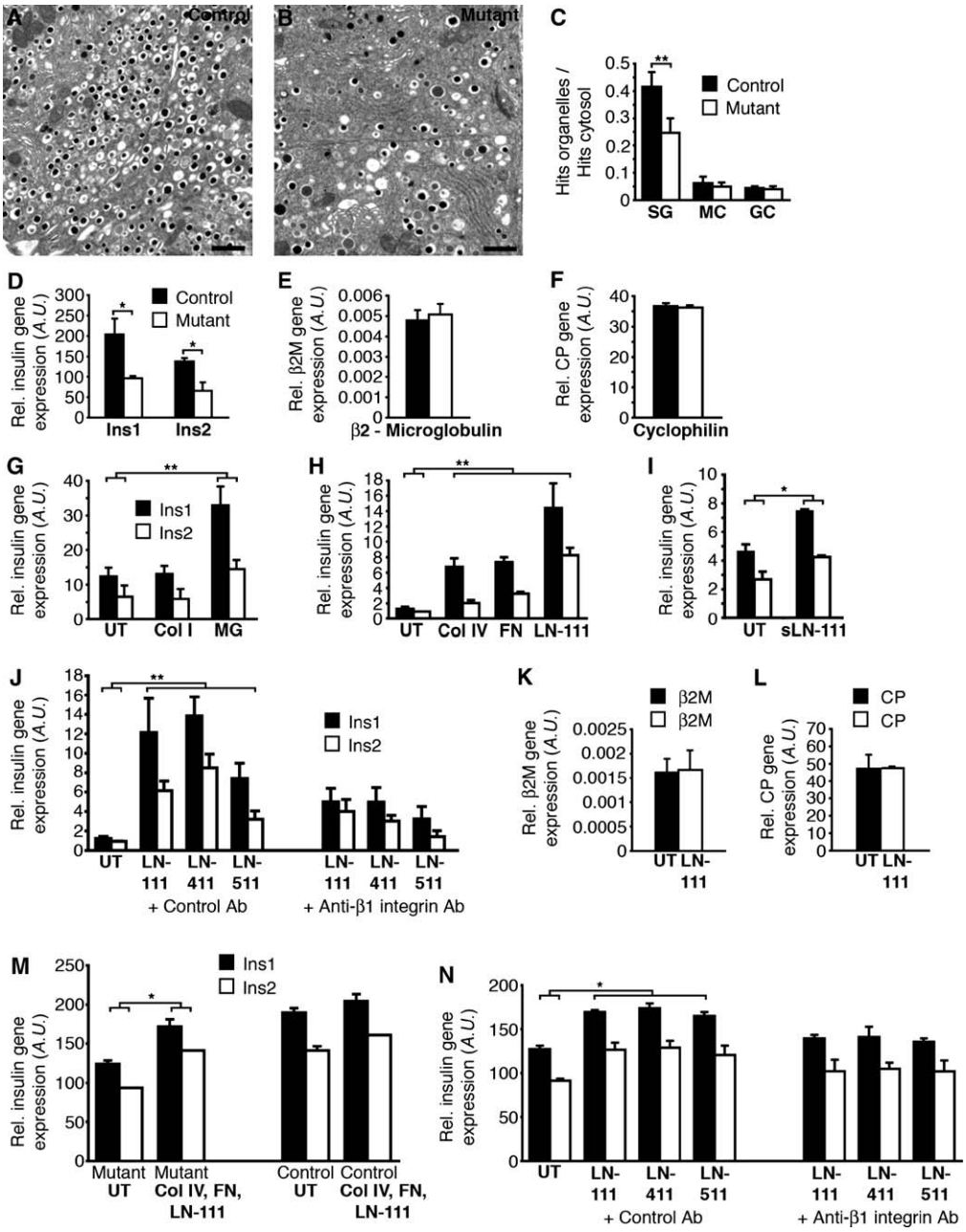

Figure 2. Regulation of Insulin Gene Expression by Signals from the Vascular Basement Membrane

(A–F) Loss-of-function experiments. (A and B) Electron microscopic images of (A) control islets and (B) mutant islets. The scale bars are 2 μm. (C) Quantification of the organelles in β 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. A total of 6–12 electron microscopic images of 5 control and 5 mutant islets each were analyzed. (D–F) Real-time RT-PCR analyses of both insulin genes (Ins1 and Ins2) as well as the control genes β2-microglobulin (β2M) and cyclophilin (CP) in mutant islets (white bars) and control islets (black bars) (n = 2 islet preparations).

(G–L) Effect of basement membrane proteins on insulin gene expression. (G and H) 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 5 μg/ml collagen IV (Col IV), fibronectin (FN), and laminin-111 (LN-111), as well as untreated plates (UT) (n = 3). (I) Relative insulin gene expression in MIN6 cells cultured in media supplemented with 30 μg/ml soluble laminin-111 (sLN-111) (n = 2). (J) Relative insulin gene expression in MIN6 cells cultured on plates coated with laminin-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). (K and L) 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).

(M and N) Rescue experiments. (M) 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). (N) 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.

*$p < 0.05$, **$p < 0.005$ (Student's t test). All values are means ± SD.

Figures 3Q and 3T with Figures 3J and 3M). This difference might be due to the presence of other α subunits in β cells (Figure 3A) that combine with β1-integrin to form alternative laminin receptors.

We confirmed the specificity of the knockdown by using two sets of siRNA molecules, siRNA-1 and siRNA-2, for each gene, which led to the same biological effects (Figures 3J, 3M, 3Q, and 3T). In addition, we showed that the knockdown of β1- and α6-integrins did not affect the expression of other integrin subunits (Figures 3H and 3O). 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 3L and 3M) (Wennerberg et al., 1996). Along with the experiments with blocking antibodies (Figures 2J and 2N), the results of the knockdown experiments demonstrate that β cells require β1-integrin for upregulating insulin gene expression in response to laminins.

Laminins Regulate Pancreatic β Cell Proliferation and Require β1-Integrin

Because the β 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 β cell proliferation (Zaret, 2004). Here, we found that the numbers of MIN6 cells in S phase increased when they were cultured on plates coated with basement membrane proteins, and that the strongest effect was induced by laminin-111 (Figure 4A). In addition, RNA interference showed that β1-integrin was required for the increased cell proliferation rate induced by this laminin (Figure 4B). Moreover, all laminins tested (laminin-111, -411, and -511) increased MIN6 cell proliferation, and anti-β1-integrin blocking antibodies extinguished this proliferative effect (Figure 4C).

To extend these findings to mouse islets, β cell proliferation rates were compared between VEGF-A-deficient mutant islets and control islets (Figure 4D). Mutant islets were smaller on average (compare Figures 1B and 1D with Figures 1A and 1C) (Lammert et al., 2003) and had fewer proliferating β cells than control islets (Figure 4E, compare the first two columns). Importantly, β cell proliferation was partially rescued when mutant islets were treated with laminin-111 (Figure 4E, compare third column with fourth column). The β cell proliferation rate also increased in control islets, but to a lesser extent when compared with mutant islets (Figures 4E). In addition, treatment of mutant islets with an anti-β1-integrin blocking antibody reduced the proliferative response of islet β cells to laminin-111 (Figure 4F). These results show that the laminins are signals from the vascular basement membrane that stimulate β cell proliferation. In addition, the proliferative effect of the laminins requires β1-integrin on β cells.

Discussion

Based on our results, we propose the following model (Figure 4G): β cells do not form a basement membrane. Instead, by using VEGF-A, they attract VEGFR2-expressing endothelial cells. These cells, in turn, form a basement membrane next to the β cells that contains laminins, collagen IV, and fibronectin. These basement membrane components were identified as endothelial signals that promote β cell proliferation and insulin gene expression to different extents. The effects of the laminins on β cells require β1-integrin. In mutant islets, in which VEGF-A is missing, no vascular basement membrane is formed next to the β cells. In these mutant islets, insulin gene expression levels and β cell proliferation rates are decreased and can be partially rescued by treatment with laminins.

It is noteworthy that the islet capsule represents an alternative, nonvascular source of laminin and collagen IV (Figures 1B and 1D). However, because of the spheroid structure of islets, most β cells are not in contact with the islet capsule, and therefore they strictly require the vascular basement membrane.

The proposed model now 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 maintain 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, overexpression of VEGF-A in islets improves their transplantation in terms of curing diabetes mellitus (Lai et al., 2005; Zhang et al., 2004).

Research on islet transplantation has shown that it takes about 1–2 weeks for transplanted islets to become revascularized 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. Based on this study, examination of whether the treatment of islets with basement membrane proteins, such as the laminins, improves islet transplantation is warranted. The results presented here suggest that laminins 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. Moreover, the observation that laminin-111 can partially substitute for endothelial cells shows that the applied laminin does not necessarily have to be an endothelial cell-derived one.

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 are required, 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).

Experimental Procedures

Laminin Nomenclature

We used the Aumailley et al. (2005) nomenclature for laminins.

Cell Culture

The MIN6 insulinoma cell line (Miyazaki et al., 1990) was used at passages 39–46. Laminin-111 (Becton Dickinson [BD]), collagen IV (BD),

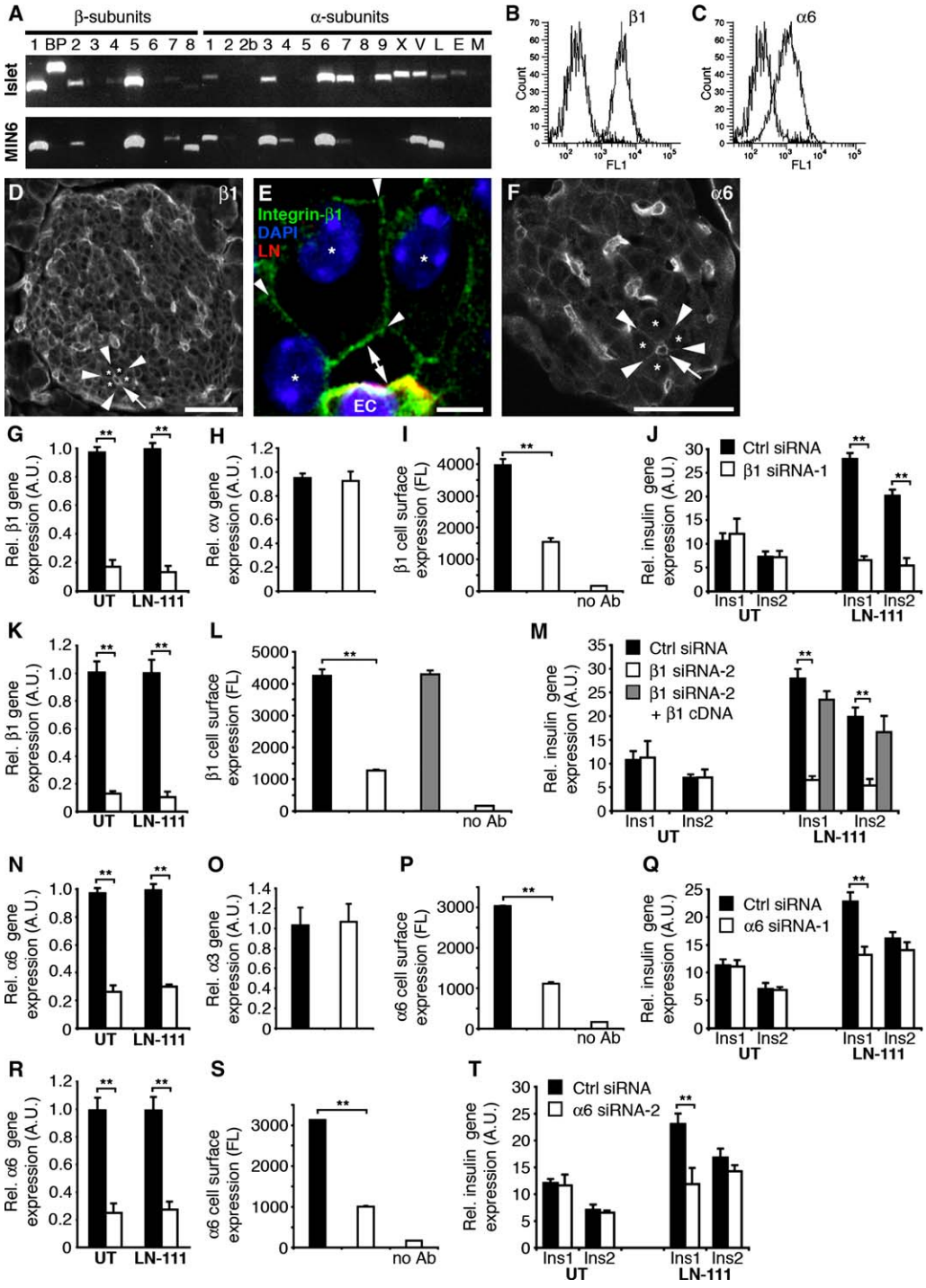

Figure 3. β1-Integrin Is Expressed by β Cells and Is Required for Upregulating Insulin Gene Expression in Response to Laminin-111

(A) Gene expression of integrin β and α subunits in islets and MIN6 cells as demonstrated by RT-PCR.

(B and 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 (D and E) β1 and (F) α6. Capillaries are labeled with arrows (in [D] and [F]), and β cell plasma membranes are labeled with arrowheads. β cells (labeled with asterisks) are often grouped as rosettes around capillaries. The artificial space (↔) between a capillary endothelial cell (EC) and a β cell in (E) was generated by high-pressure perfusion to illustrate β1-integrin expression on the β cell plasma membrane facing the LN-expressing EC. Scale bars in (D) and (F) are 50 μm, and the scale bar in (E) is 5 μm.

(G–M) β1-integrin knockdown experiments. The black bars represent MIN6 cells transfected with Ctrl siRNA, the white bars represent MIN6 cells transfected with either (G–J) β1 siRNA-1 or (K–M) β1 siRNA-2, and the gray bars represent β1 siRNA-2-transfected MIN6 cells rescued by the β1 cDNA. (G and K) β1-integrin gene expression in MIN6 cells cultured on untreated plates (UT) and laminin-coated plates (LN-111). (H) Integrin αv gene expression in β1 siRNA-1-transfected MIN6 cells. (I and L) β1-integrin cell surface expression; FL, mean fluorescence. (J and M) Insulin (Ins1 and Ins2) gene expression in MIN6 cells grown on untreated (UT) and laminin-coated plates (LN-111).

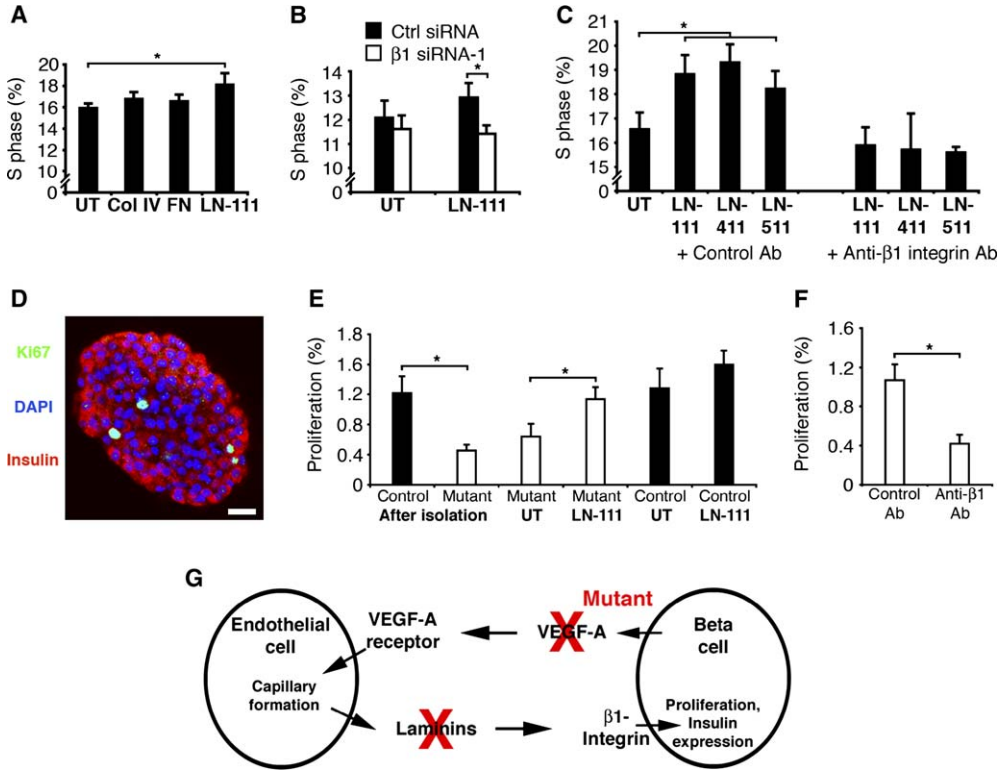

Figure 4. Regulation of Pancreatic β Cell Proliferation by Laminins and Requirement for β1-Integrin

(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)–(C) were abbreviated.

(D–F) β cell proliferation defect in mutant islets and rescue by laminin-111. (D) Isolated islets were stained for Ki67 (green), insulin (red), and nuclei (blue). Cells coexpressing Ki67 and insulin were counted as proliferating β cells. The scale bar is 20 μm. (E) β cell proliferation (as a percentage of Ki67-positive β 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). (F) β 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 (E) and (F) are means ± SEM.

(G) Proposed model (see Discussion).

fibronectin (Upstate), recombinant laminin-411 (LN-411) (Kortesmaa et al., 2000), and recombinant laminin-511 (LN-511) (Doi et al., 2002) were used at a concentration of 5 μg/ml for coating non-tissue culture-treated 6-well plates (Falcon). Coated and untreated (UT) plates were blocked with 1% BSA (Sigma). Hamster IgM and hamster anti-rat CD29 IgM (Ha2/5) (BD) were used as control and anti-β1-integrin blocking antibody, respectively (BD). Collagen type I (BD) as well as growth factor-reduced matrigel (BD) were used as 5 mm thick gels. Cells were incubated for 3–4 days.

Pancreatic Islet Culture

Pancreatic islets were cultured for 3 days in medium alone (UT) or in medium containing 10 μg/ml Col IV, 10 μg/ml FN, and 30 μg/ml LN-111, or 30 μg/ml LN-111, LN-411, or LN-511. The mRNAs were isolated either directly after islet isolation or after 3 days of incubation. For assessment of β cell proliferation, islets were cultured for 3 days in medium alone (UT) or in medium containing 50 μg/ml LN-111.

Gene Silencing and RT-PCR

One million MIN6 cells were electroporated with 5 μg siRNA directed against the β1- and α6-integrins, as well as firefly luciferase (Ctrl siRNA). For every RT-PCR experiment, 1 μg MIN6 total RNA and 500 ng islet total RNA were used. All integrin primers were tested on cDNA pooled from different mouse embryonic and adult organs (positive controls). For each real-time RT-PCR experiment (SYBR green method), 80 ng MIN6 total RNA and 40 ng islet total RNA were used. Each sample was run in duplicate, and "no template controls" were included for each primer pair (see the Supplemental Data available with this article online). Data were analyzed according to the threshold cycle method (Ct). Comparison of the α-tubulin

(N–T) α6-integrin knockdown experiments. The black bars represent MIN6 cells transfected with Ctrl siRNA, and the white bars represent MIN6 cells transfected with either (N–Q) α6 siRNA-1 or (R–T) α6 siRNA-2. (N and R) α6-integrin gene expression in MIN6 cells cultured on untreated plates (UT) and laminin-coated plates (LN-111). (O) α3-integrin gene expression in α6 siRNA-1-transfected MIN6 cells. (P and S) α6-integrin cell surface expression; FL, mean fluorescence. (Q and T) 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.

mRNA levels with the cyclophilin and β2-microglobulin mRNA levels in MIN6, control, and mutant islets validated the use of α-tubulin as an internal control.

Antibodies, Microscopy, and FACS

The following primary antibodies were used: rabbit anti-laminin (EHS) (Sigma); rabbit anti-laminin subunits α1 (Durbeej et al., 1996), α4, and α5 (Sixt et al., 2001); rabbit anti-collagen IV (Chemicon); rat anti-PECAM1 (BD Pharmingen); rat anti-β1-integrin (Chemicon); rat anti-α6-integrin (BD Pharmingen); rabbit anti-Ki67 (Novocastra Laboratories Ltd.); and guinea pig anti-insulin (DAKO). Ki67-positive β cells and DAPI-positive nuclei were counted in Z-sections taken from whole islets. Electron microscopy of islets and quantification of their insulin secretory granules, mitochondria, and Golgi complexes were performed as recently described (Lammert et al., 2003). The mean fluorescence of β1- and α6-integrin antibody-stained MIN6 cells and cell cycle phase distribution were analyzed by using FACS and CELLQuest software (BD).

Mouse Islet Cell Isolation

Mutant mice (Pdx1-Cre × VEGFloxP) expressed 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 gene expression and immunohistochemical analyses of the laminin α chains and integrins, 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. A total of 2 ml 0.23 mg/ml Liberase RI solution was injected into the bile duct, followed by digestion at 37ºC 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 were used for RNA isolation, immunohistochemistry, islet culture, or islet cell isolation. In order to separate the islet endothelial and β cell populations, islets were dissociated with trypsin-EDTA. Rat anti-PECAM1 antibody and Cy5-conjugated donkey anti-rat secondary antibody were used for labeling islet endothelial cells. Autofluorescent β cells and antibody-labeled endothelial cells were isolated with FACSAria (BD), and both populations were used for conventional RT-PCR.

Statistical Analyses

All values were expressed as means ± SD, except for those in Figures 4E and 4F, which showed 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 $p < 0.05$.

Supplemental Data

Supplemental data including siRNA sequences, real-time RT-PCR primers, and references are available at http://www.developmentalcell.com/cgi/content/full/10/3/397/DC1/.

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