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Binding of the fibronectin-mimetic peptide, PR_b, to $\alpha_5\beta_1$ on pig islet cells increases fibronectin production and facilitates internalization of PR_b functionalized liposomes

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Abstract

Islet transplantation is a promising treatment for type 1 diabetes. Recent studies have demonstrated that human islet allografts can restore insulin independence to patients with this disease. As islet isolation and immunotherapeutic techniques improve, the demand for this cell-based therapy will dictate the need for other sources of islets. Pig islets could provide an unlimited supply for xenotransplantation and have shown promise as an alternative to human islet allografts. However, stresses imposed during islet isolation and transplantation decrease islet viability, leading to loss of graft function. In this study, we investigated the ability of a fibronectin-mimetic peptide, PR_b, which specifically binds to the $\alpha_5\beta_1$ integrin, to reestablish lost extracellular matrix (ECM) around isolated pig islets and increase internalization of liposomes. Confocal microscopy and western blotting were used to show the presence of the integrin $\alpha_5\beta_1$ on the pig islets on day 0 (day of isolation), as well as different days of islet culture. Islets cultured in medium supplemented with free PR_b for 48 hours were found to have increased levels of ECM fibronectin secretion compared to islets in normal culture conditions. Using confocal microscopy and flow cytometry we found that PR_b peptide-amphiphile functionalized liposomes delivered to the pig islets internalized into the cells in a PR_b concentration dependent manner, and non-functionalized liposomes showed minimal internalization. These studies proved that the fibronectin-mimetic peptide, PR_b, is an appropriate peptide bullet for applications involving $\alpha_5\beta_1$ expressing pig islet cells. Fibronectin production stimulated through $\alpha_5\beta_1$ PR_b binding may decrease apoptosis and therefore increase islet viability in culture. In addition, PR_b peptide-amphiphile functionalized liposomes may be used for targeted delivery of different agents to pig islet cells.

Introduction

Type 1 diabetes continues to present therapeutic challenges. Recent results have shown the potential of islet transplantation as an alternative to whole pancreas transplantation for diabetes treatment¹. Several programs have reported successful restoration of normoglycemia and insulin independence in immunosuppressed patients who received

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Supporting Information Available: Confocal image of fibronectin production with 100Kx PR_b, fibronectin confocal images quantification, table with liposome characterization data, and additional flow cytometry experimental data are located in the Supporting Information. This information is available free of charge via the Internet at <http://pubs.acs.org>.

human islet allografts.²⁻⁵ As of 2005, 82% of patients receiving human islet allografts at the three leading islet transplantation centers were insulin independent at one year post islet transplant completion.^{6, 7} As this cell-based therapy benefits from improved isolation and immunotherapeutic techniques, the demand for islet replacement therapy will increase, straining the already short supply of human islet donors.⁸ Porcine islets could provide an unlimited supply for islet transplantation and have shown promise as a successful alternative to human islet allografts.⁸⁻¹⁴ Porcine islets are advantageous due in part to the on-demand availability of young, healthy, living, pathogen-free donors, and the ease of breeding.^{8, 15} Additionally, porcine islets respond to blood glucose concentration similarly to human islets and porcine insulin has been used for daily injections, differing from human insulin by only one amino acid.¹⁶⁻¹⁹

The isolation process affects both human and porcine islet cell viability.^{20, 21} The loss of viability stems, at least in part, from the loss of extracellular matrix (ECM) from the islets' environment.^{20, 22, 23} Adhesion to ECM is mediated through integrin receptors. $\alpha_5\beta_1$ is an important integrin for promoting cell adherence, spreading, survival, and angiogenesis.²⁴⁻²⁸ It binds the Arg-Gly-Asp (RGD) sequence of ECM proteins including fibronectin, vitronectin, and fibrinogen.^{29, 30} Fibronectin also contains a synergy sequence Pro-His-Ser-Arg-Asn (PHSRN) that contributes to the high affinity of the $\alpha_5\beta_1$ integrin to fibronectin.³¹⁻³⁴ Regarding islets, various integrins have been studied to determine their role in the development of the pancreas and in islet survival.^{20, 22, 23, 35-37} The integrin repertoire displayed on islets from human^{35, 36}, hamster, porcine³⁶, canine²³, and rat³⁷ lines have been investigated by various groups. Wang et al.³⁶ studied porcine islets and found expression of integrin subunits α_2 , α_3 , α_5 , and α_v but not β_1 .

RGD peptides are commonly used as bullets for targeting integrins including $\alpha_5\beta_1$.^{38, 39} However, RGD peptides do not have the same binding strength and affinity as native fibronectin^{40, 41} thus limiting their therapeutic use. Previously, we hypothesized that a peptide mimicking both the distance and hydrophilicity/hydrophobicity between the primary binding RGD domain and the PHSRN synergy site on fibronectin could increase the peptide's binding affinity and specificity to $\alpha_5\beta_1$.^{42, 43} In native fibronectin the distance between RGD and PHSRN is 30-40 Å.⁴⁴ We sought to mimic this distance and the relative hydrophilicity/hydrophobicity of the region spanning the two sequences. We designed a peptide, PR_b, composed of a spacer (KSS) at the N terminus, the fibronectin synergy site sequence (PHSRN), a linker ((SG)₅) and the primary binding sequence (RGDSP).⁴² The length of the linker is 37 Å and the hydrophilicity/hydrophobicity ratio is 1:1; one hydrophobic glycine (G) to one hydrophilic serine (S) amino acid, reflecting the approximate ratio found in native fibronectin.^{42, 43} PR_b was synthesized as a peptide-amphiphile by conjugating it to a dialkyl tail (Figure 1). The design features the PR_b headgroup, a C₁₆ dialkyl ester tail with a glutamic acid (Glu) tail linker and -(CH₂)₂- tail spacer. The (C₁₆)₂-Glu-C₂ was connected to the N terminus of PR_b. Our original hypothesis stated that the degree of hydrophilicity/hydrophobicity is an important design parameter for a fibronectin-mimetic peptide. We recently compared the PR_b peptide-amphiphile to similar peptide-amphiphiles containing the RGD and PHSRN sequences spanned by a purely hydrophobic or purely hydrophilic region.⁴⁵ We found that PR_b outperformed the other peptide sequences in binding strength and human umbilical vein endothelial cell (HUVEC) adhesion. We have also compared surfaces functionalized with PR_b to surfaces functionalized with GRGDSP, 50 mol% GRGDSP- 50 mol% PHSRN randomly presented at the interface, and native fibronectin. We demonstrated that the PR_b functionalized surfaces outperformed fibronectin and other peptide surfaces in terms of HUVEC cell adhesion, cell spreading, $\alpha_5\beta_1$ -specificity, cytoskeleton organization, and ECM fibronectin production.⁴² The PR_b peptide-amphiphile (PR_b PA) was also used for targeted drug delivery⁴⁶⁻⁴⁸ and tissue engineering applications.⁴⁹ PR_b PA functionalized

conventional and stealth liposomes (liposomes covered with polyethylene glycol, PEG) showed improved cell binding and internalization as compared to non-targeted liposomes or liposomes functionalized with GRGDSP in prostate cancer cells⁴⁶ and colon cancer cells.⁴⁷ We have demonstrated specificity of PR_b to $\alpha_5\beta_1$ by blocking cell adhesion with antibodies⁴² and liposomal cell internalization with free PR_b.⁴⁷

Losses of porcine islet viability during isolation and after transplantation are major problems in xenotransplantation of the fresh porcine islets. The goal of this study was to examine $\alpha_5\beta_1$ expression on porcine islet cells and determine if PR_b is an appropriate peptide for targeting islets in culture. We hypothesized that the PR_b peptide could increase the production of ECM proteins, such as fibronectin, in cultured islets, thus reestablishing the ECM that was lost during digestion. We also hypothesized that PR_b PA functionalized liposomes would show increased internalization into porcine islet cells thus providing a possible mechanism for enhanced delivery of encapsulated agents of interest for cytoprotecting, immunomodulating, as well as imaging of cultured porcine islets after transplantation. We therefore envision a scenario in which PR_b could be used in a multi-faceted approach to protecting and maintaining islet viability. Free PR_b peptide would be used to target islets soon after isolation, promoting ECM production, and thus preventing apoptosis and increasing islet yield. Then, PR_b PA functionalized liposomes would be targeted to islets *ex vivo* to deliver molecules that could protect the islets from hypoxic and other stresses encountered after transplantation or allow for monitoring viability and imaging islets after transplantation. In this paper we first present evidence of $\alpha_5\beta_1$ integrin expression on the porcine islet cells on the days following islet isolation for the first time in literature. We go on to show increased fibronectin production from porcine islets cultured with supplemented PR_b. Finally, our data show that PR_b PA functionalized liposomes internalize into porcine islets in a concentration dependent manner while non-targeted liposomes do not internalize. While we do not present an application in this study, we are currently investigating multiple uses of this technology for use in porcine islet culture.

Materials and Methods

Porcine Islet Isolation and Oxygen Consumption Rate Analysis

Porcine islets were isolated and cultured free floating as previously described.⁵⁰⁻⁵³ Islet quality control⁵⁴ revealed $1,789 \pm 1,194$ islet equivalents (IE)/g pancreas, corresponding to an average islet yield of $92\% \pm 35\%$ IE. IE is a spherical aggregate of cells with a 150 μm mean diameter.⁵⁵ The purity of the islet graft as assessed by the percentage of dithizone-positive cells was $>90\%$ (mean, 94%). For all experiments, the number of IE was determined based on IE availability and number of IE required to perform the experiment. Oxygen consumption rate (OCR) analysis was used to determine islet viability and was performed as described previously.^{56, 57} The OCR/DNA on day 7 ranged from 71-276 nmol/min-mg DNA. OCR analysis was also used to evaluate PR_b cytotoxicity. The OCR/DNA ratio of islets exposed to 0.1 mg/mL PR_b for 24 or 72 hours of culture was compared to control islets at those time points. Data were obtained from 9 pig isolations.

Integrin Expression

Approximately 100 IE were removed from culture for each sample, pelleted in microcentrifuge tubes by centrifugation at 800 rpm for 1 minute in a tabletop centrifuge, and washed twice with phosphate buffered saline (PBS). Samples were incubated at 4°C with primary antibodies to $\alpha_5\beta_1$ (JBS5), α_5 (P1D6), and β_1 (HB1.1) (Millipore, Temecula, CA) at a 1:33 dilution over a rotary shaker for 1 hour. Islets were pelleted and washed twice in PBS, incubated at 4°C with secondary antibody, anti-mouse IgG FITC-conjugated (Millipore), at a 1:33 dilution over a rotary shaker for 1 hour, pelleted and washed twice. Nuclear staining

was performed using a cell membrane permeable blue-fluorescent Hoechst 33342 dye (Molecular Probes, Eugene, OR) at a concentration of 2.0 μ M and membrane staining was performed using a cell-impermeable red-fluorescent Alexa Fluor 594 wheat germ agglutinin (WGA; Molecular Probes) at 5.0 μ M in PBS for 45 minutes at room temperature over a rotary shaker. Islets were washed twice, resuspended in PBS and prepared for immediate confocal microscopy analysis. The Olympus Fluoview FV1000 confocal laser scanning microscope at the Biomedical Image Processing Laboratory at the University of Minnesota was used for all confocal studies. 10 to 15 z-scans (horizontal cross-section of the islet at a particular z height) were taken for each islet. Images shown are z-scans from the middle of the islet. The $\alpha_5\beta_1$ expression was analyzed on the specified days of culture for at least two different porcine islet isolations.

Integrin expression was also determined using western blot. Protein lysate from multiple isolated islet lots were received from the Schulze Diabetes Institute. Protein concentration was determined by the BCATM Protein Assay Kit (Thermo Scientific, Waltham, MA) following the manufacturer's protocols. Samples were separated by 7.5% SDS-PAGE under non-reducing conditions, transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad), and analyzed by western blotting. Antibodies to α_5 (AB1949) and β_1 (AB1952), and the secondary antibody anti-rabbit horseradish peroxidase (HRP) conjugate (12-348) were purchased from Millipore. Blots were developed using a TMB (3,3',5,5'-tetramethylbenzidine) Stabilized Substrate for Horseradish Peroxidase (Promega, Madison, WI).

Fibronectin Production

Approximately 100 IE were removed from culture for each sample and suspended in 2 mL of warm culture medium (Medium 199 (Sigma) supplemented with 10% donor pig serum and ciprofloxacin) in disposable plastic dishes (BD Biosciences, San Jose, CA). Free PR_b peptide (received from the BioMedical Genomics Center at the University of Minnesota) dissolved in PBS was delivered to the islets at specific theoretical excesses as determined by the following calculation: Assuming an upper limit of 50,000 $\alpha_5\beta_1$ integrins per cell⁵⁸ and approximately 1,500 cells per IE,⁵⁵ there are a total of 7.5×10^7 $\alpha_5\beta_1$ integrins per IE. Therefore 1.25×10^{-16} moles of PR_b is necessary per islet for a 1:1 ratio of $\alpha_5\beta_1$ to PR_b. We gave concentrations of PR_b peptide at 5,000 (5Kx; 133 ng PR_b), 10,000 (10Kx; 267 ng), 20,000 (20Kx; 534 ng), and 100,000 (100Kx; 2,670 ng) times excess this theoretical 1:1 amount. The PR_b was added to the culture medium on the indicated day and allowed to incubate for 48 hours. After 48 hours the islets were stained with anti-fibronectin primary antibody specific for secreted fibronectin (PIH11, 1:133 dilution, Millipore) for 1 hour at 4°C over a rotary shaker and then stained with a secondary antibody (anti-mouse IgG FITC conjugated; 1:133 dilution) for 1 hour at 4°C. Nuclear and membrane staining were performed as described in the integrin expression section. Confocal images were taken immediately after sample preparation. 10 to 15 z-scans were taken for each islet. The FluoView software was used to create 2D z-projections of the optical sections. This image analysis technique projects the fluorescent features in each z-scan of the islet onto a common image plane positioned parallel to the source slices. The result is a single 2D composite representation of the fluorescent features from all slices included in the projection.

Fibronectin production was also analyzed using western blotting. After incubation for 48 hours with or without free PR_b in serum-free medium, the islet medium was collected and filtered three times for 30 minutes each with a 10 KD centrifugal filter device (Millipore) at 4 °C. Filtration was necessary to remove excess free PR_b peptide present in the medium. Protein concentration was determined as above and 20 μ g of protein was loaded into the gels. The samples were separated by SDS-PAGE under reducing conditions and transferred

to a PVDF membrane. Fibronectin was detected with an anti-fibronectin antibody (AB1945, Millipore), followed by an anti-rabbit HRP conjugate secondary antibody. Membranes were developed using TMB Stabilized Substrate for HRP and quantified using Quantity One software (Bio-Rad).

Liposome Preparation and Characterization

The PR_b peptide-amphiphile (PR_b PA) ((C₁₆)₂-Glu-C₂-KSSPHSRN(SG)₅RGDSP) (PR_b headgroup KSSPHSRN(SG)₅RGDSP was purchased in crude form from the BioMedical Genomics Center at the University of Minnesota) was synthesized as described previously.⁴³ Liposomes composed of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), cholesterol (Avanti Polar Lipids Inc., Alabaster, AL), and PR_b PA were prepared as described elsewhere.⁵⁹ Briefly, DPPC, cholesterol, and PR_b PA solutions were combined at concentrations of *x* mol% PR_b PA, 35 mol% cholesterol, and (65-*x*) mol% DPPC. The liposomes were hydrated with 2 mM of calcein (excitation 494, emission 517) (Invitrogen, Carlsbad, CA) in HBSE buffer (10 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, pH 7.4) at 65°C and at a final lipid concentration of 10 mM. The hydrated lipids were freeze-thawed five times and then extruded through two stacked 100 nm polycarbonate membranes (Avestin Inc, Ottawa, Canada) 21 times using a hand-held extruder (Avestin). Liposomes were filtered over a Sepharose CL-4B gel filtration column. Filtered liposomes were stored at 4-8°C for up to 2 weeks before use. Phospholipid concentration was determined using the phosphorus colorimetric assay described elsewhere⁶⁰ and total PR_b PA concentration (on the inside and outside of the liposomes) was determined using the BCA assay according to the manufacturer's protocol. The ZetaPALS Zeta Potential Analyzer (Brookhaven Instruments, Holtsville, NY) was used to determine both the zeta potential (electric potential at the interfacial double layer of the liposome) and diameter of the liposomes by dynamic light scattering (DLS). Cryogenic-transmission electron microscopy (Cryo-TEM) was used for direct imaging of PR_b functionalized liposomes. Vitrobot (FEI company) was used to prepare the Cryo-TEM samples. 3 μL of liposome solution was put on lacey Formavar/Carbon grid. The grid was blotted for 1 second with an offset of -2. The specimen was immediately immersed into liquid ethane cooled close to its freezing point. The vitrified specimen was transferred, without rewarming, into a Gatan 613.DH cooling holder and observed on a JEOL 1210 TEM operated at 120 kV.

Liposome Internalization and Binding

For analysis of liposome internalization, approximately 150 IE were used per sample. The islets were removed from culture on the indicated day and suspended in 0.5 mL of culture medium. Liposomes were added at 250 μM lipid concentration and the islets were incubated at 37°C over a rotary shaker for the specified time periods (24 or 48 hours). After incubation, the islets were pelleted and washed twice. The islets were then fixed in 4% paraformaldehyde in PBS (pH 7.4) for 15 minutes at 37°C. Cell nucleus and membrane staining were performed as described in the integrin expression section. Confocal images were taken immediately after sample preparation. 10 to 15 z-scans were taken for each islet. The FluoView software was used to create 2D z-projections of the optical sections as described above. Each treatment (day removed from culture, mol% PR_b PA, and length of incubation) was examined for at least two different islet isolations. For flow cytometric analysis, approximately 800 IE were used per sample. The islets were suspended in 1.5 mL of culture medium. Liposomes were added at 250 μM lipid concentration and the islets were incubated at 37°C over a rotary shaker for 24 hours. After incubation, the islets were pelleted and washed twice with Buffer A (0.5% bovine serum albumin (BSA) (Sigma), 2mM ethylenediaminetetraacetic acid (EDTA) in PBS). The islets were dissociated into a single cell suspension by incubating at 37°C with 1 mL of cell dissociation buffer (Sigma) over a rotary shaker, pelleted and washed twice with Buffer A. The dissociated islets were

resuspended in PBS and flow cytometric analysis was carried out immediately on a FACS Calibur instrument located at the Flow Cytometry Core facility in the Cancer Research Center of the University of Minnesota.

Results and Discussion

Integrin Expression

Porcine islet cells were stained for integrin subunits α_5 and β_1 and the $\alpha_5\beta_1$ complex on day 0 (day of isolation), day 1, day 2, day 3, day 7 and day 11 of culture. Intact islets were examined by confocal microscopy with 10-15 z-scans per islet. The images shown in Figures 2 and 3 are z-scans taken from the middle of the islet. Figure 2 A-F shows expression of $\alpha_5\beta_1$ on all days examined with no apparent trend in expression. Figure 3 shows expression of the individual α_5 (A, C) and β_1 (B, D) subunits on days 0 and 11 of culture. Protein lysates from porcine islets during days 0, 2, and 7 or 8 of culture were used to confirm the expression of the α_5 and β_1 subunits via Western blotting. These results are shown in Figure 3E. Under non-reducing condition α_5 runs at 155 kDa and β_1 at 110 kDa. These bands are apparent on the respective blots. The 220 kDa band on the β_1 blot represents a dimer of the β_1 subunits,⁶¹ while the band at 150 kDa represent the β_1 chain associated with an α chain, seen previously in other reports.^{62, 63} The α_5 and β_1 subunits have been shown to be expressed on islet cells from different species,^{23, 35-37} and α_5 has been shown to be expressed on porcine islets,³⁶ but we are the first to show the presence of β_1 on porcine islet cells.

Fibronectin Production

Incorporating ECM mimetic peptides into cellular environments has been shown to increase matrix production and cell viability.^{37, 64-66} Since $\alpha_5\beta_1$ was constitutively expressed on the cultured islet cells, PR_b (a fibronectin-mimetic peptide that specifically binds to $\alpha_5\beta_1$ was an excellent candidate for targeting these integrins. Our previous research demonstrated that PR_b has stronger adhesion to $\alpha_5\beta_1$ than the commonly used GRGDSP peptide or other fibronectin-mimetic peptides.^{42, 45} Additionally, we found HUVECs attached to surfaces fully functionalized with PR_b had increased fibronectin production compared to other peptide and protein surfaces.⁴² Therefore, we hypothesized that the addition of free PR_b peptide to the islet culture would increase ECM production, restoring the matrix lost during the isolation process. To ensure that PR_b is not cytotoxic to the islet cells OCR analysis was performed on islets isolated from nine different pigs that were cultured with or without 0.1 mg/mL PR_b for 24 or 72 hours. The presence of dead cells lowers the OCR/DNA ratio. Figure 4 shows that the OCR/DNA ratio was similar for islet cells cultured with or without PR_b at both time points demonstrating that PR_b is not cytotoxic to the islet cells.

To determine if PR_b can reestablish the production of ECM after islet isolation we added free PR_b to islet samples during the culture period. Porcine islets do not attach to the culture flask so integrin binding is dependent only on ligands present in the medium. The concentrations of PR_b employed were determined by calculating a hypothetical amount of PR_b necessary to bind to a theoretical number of $\alpha_5\beta_1$ integrins on the islet cells, assuming one molecule of PR_b binds to one integrin. PR_b was added to the culture medium in amounts excess of this 1:1 concentration. PR_b was supplemented at 5,000 (5Kx), 10,000 (10Kx), 20,000 (20Kx), and 100,000 (100Kx) times excess this theoretical concentration to the culture medium of the islet samples on day 2 of culture after isolation (day 0) and allowed to incubate for 48 hours before the islets were analyzed with confocal microscopy. The results are shown in Figure 5 and are 2D z-projections of all the fluorescent features from the multiple layers of the islet that were captured. Control samples (Figure 5A) showed minimal fibronectin production at the periphery. Islets incubated with 5Kx (Figure 5B)

showed fibronectin production primarily at the periphery of the islet, while islets incubated with increased peptide concentrations, 10Kx (Figure 5C) and 20Kx (Figure 5D), showed fibronectin production within the core of the islet as well as at the periphery. This result is representative of all samples analyzed. Confocal images were quantified and results are shown in Figure S1 (Supporting Information). The 20Kx PR_b promoted the highest level of fibronectin production. Adding 100Kx (Figure S2 in Supporting Information) did not further increase the fibronectin production levels possibly because the $\alpha_5\beta_1$ integrins were fully saturated. Free PR_b at the same concentrations (5Kx, 10Kx, and 20Kx) were also added to islets on day 0 and allowed to incubate for 48 hours for western blotting analysis. The medium from these samples was analyzed to quantify the amount of fibronectin present in the medium. The blot shown in Figure 6 demonstrates that increasing PR_b leads to more fibronectin production, and that 20Kx produces significantly more fibronectin than the control or 5Kx samples. These trends are similar to the trends observed by quantifying confocal images of PR_b supplemented islets (Figure S1). Together these results show that the PR_b peptide induces increased fibronectin production in free floating islets. Fibronectin production is beneficial for islets because it reestablishes integrin-matrix interactions which provide survival signals to the cell.^{27, 29} Analyzing the effects of this increased fibronectin production on the viability of the porcine islets in culture is the subject of future work.

Liposome Characterization and Internalization

Targeted drug delivery has many applications from disease treatment to imaging applications.⁴¹ We investigated the ability of PR_b PA functionalized conventional liposomes to bind and internalize into porcine islet cells. The use of liposomes to deliver agents of interest to islet cells in culture before transplantation could increase the delivery efficiency and lower the total amount of agent needed for the specific application. We hypothesized that by functionalizing the liposomes with PR_b PA we could increase internalization of conventional liposomes. We investigated whether conventional liposomes with no peptide or functionalized with different amounts of PR_b PA could bind and internalize in porcine islet cells in culture. Liposomes were characterized via DLS, zeta potential measurements, and cryo-TEM. We studied liposome internalization for a variety of liposome formulations, days of culture for islets, and incubation periods for liposomes. Since PR_b PA is the targeting bullet, the final mole percent of PR_b PA in the liposomes is used to distinguish the different liposome formulations. The liposome formulations used in this study are shown in Table S1 (Supporting Information). Differences in size and zeta potential don't seem to play a role in the level of liposome internalization, therefore any differences in binding and internalization should stem from the amount of PR_b PA used to functionalize the liposomes. Figure 7 shows a cryo-TEM image of liposomes functionalized with 1.6 mol% PR_b PA. The image shows a mean diameter of about 100 nm which is consistent with DLS data shown in Table S1.

The liposomes were delivered to islets on the day of isolation (day 0) or on day 2 of culture and incubated for 24 or 48 hours. Figure 8 shows representative images of the liposome and time combinations. Liposomes with no PR_b PA (0% PR_b PA) showed no internalization in the islet cells. With 0.6 mol% of PR_b PA functionalization there was increased internalization and even more seen for the 1.6 mol% and 3.2 mol% PR_b PA formulations. Liposomes functionalized with 3.2 mol% PR_b PA had the greatest level of internalization after 48 hours of incubation. As shown in the figure, the internalization efficiencies depended on the PR_b PA concentration and the time of incubation which has been seen previously in the targeted delivery of PR_b PA functionalized liposomes to cancer cells.^{46, 47} Greater internalization was also seen after 48 hours of incubation as compared to 24 hours. The images are 2D z-projections of the fluorescent features from the multiple layers of the islet that were captured with the confocal microscope.

Flow cytometry was used to quantify the level of binding and internalization of the PR_b PA functionalized and non-functionalized liposomes. The flow cytometry data shown in Figure 9 are from liposomes delivered to islets on day 0 (day of isolation) and analyzed after 24 hours of incubation. The figure shows a concentration dependent effect, where liposomes without PR_b PA show minimal binding to the islet cells and liposomes functionalized with 0.6, 1.0, and 3.2 mol% PR_b PA showed increased levels of fluorescence. The table in Figure 9 shows the percentage of islet cells with a positive shift in fluorescence compared to the control samples. From the data, the PR_b PA concentration dependent increase in cell binding is clear; higher PR_b PA functionalization leads to a greater percentage of cells with a positive shift in fluorescence. This trend was also seen for liposomes delivered to islets on days 7 and 12 of culture (Figure S3 in Supporting Information). Data show that PR_b PA functionalized liposomes have improved cell binding and internalization ability compared to non-functionalized liposomes when delivered to porcine islets of Langerhans. These results confirm that PR_b PA functionalized liposomes could be used to deliver agents of interest to the porcine islets in culture.

Conclusions

In this study we demonstrated the presence of the $\alpha_5\beta_1$ integrin on porcine islet cells. These studies also show that the fibronectin-mimetic peptide, PR_b, is an appropriate peptide bullet for applications involving porcine islets. The free peptide was shown to stimulate ECM production from isolated islet cells, reestablishing integrin-matrix connections lost during isolation. PR_b PA was also shown to increase internalization of conventional liposomes into islet cells, potentiating their use for delivering agents of interest *ex vivo* to porcine islet cells. Our lab is currently investigating a number of methodologies for which PR_b may play a critical role in protecting, maintaining, and tracking porcine islets during isolation, the culture period, and after transplantation. These studies will be the subject of future work.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Schematic of PR_b peptide-amphiphile

Figure 2.

Porcine islets of Langerhans stained for $\alpha_5\beta_1$ (green), nucleus (blue), and cell membrane (red) on different days. $\alpha_5\beta_1$ was stained on **A** the day of isolation (D 0), **B** day 1 of culture (D 1), **C** day 2 of culture (D 2), **D** day 3 of culture (D 3), **E** day 7 of culture (D 7), and **F** day 11 of culture (D 11). The images are z-scans from the middle of the islet. All scale bars are 20 μm .

Figure 3.

Expression of α_5 and β_1 on porcine islets of Langerhans characterized by confocal microscopy and Western blotting. Porcine islets were stained for α_5 or β_1 (green), nucleus (blue), and cell membrane (red) on different days of culture and imaged by confocal microscopy. α_5 was stained on **A** day 0 and **C** day 11 of culture. β_1 was also stained on **B** day 0 and **D** day 11 of culture. Separate islet samples were used to stain α_5 and β_1 so there is no colocalization of the subunits in the images. The images are z-scans from the middle of the islet. All scale bars are 20 μm . **E** Western blots showing expression of the integrin subunits α_5 and β_1 in porcine islet cells on days 0, 2, and 7 or 8 of culture. The molecular weight marker is shown to the right of each image.

Figure 4. Oxygen consumption rate (OCR) per mg DNA for islet cells incubated with 0.1 mg/mL free PR_b (+ PR_b) or without PR_b (-PR_b) for 24 or 72 hours of culture. The experiment was performed on islets from nine isolations (n=9). Data are expressed as the mean $\pm$ standard deviation.

Figure 5.

PR_b was added to the culture medium on day 2 of culture and the islets were imaged 48 hours later by confocal microscopy. Images show the cell membrane (red), nucleus (blue), and secreted fibronectin (green). The images are 2D z-projections of the fluorescent features from the multiple layers of the islet that were captured. The amount of PR_b added to the culture medium is as follows: **A** Control- no PR_b was added to the culture medium, **B** 5Kx (133 ng), **C** 10Kx (267 ng), **D** 20Kx (534 ng). All scale bars are 20 μm.

Figure 6.

PR_b was added to serum-free culture medium on day 0 of islet culture and allowed to incubate for 48 hours. The resulting medium was analyzed by western blotting. The amount of PR_b added to the culture medium is as follows: Ctrl (no PR_b was added to the culture medium), 5Kx (133 ng), 10Kx (267 ng), and 20Kx (534 ng). Data in the graph are expressed as the mean $\pm$ standard deviation of band densities from two western blots. Student t-test statistical analysis shows a significant difference between both the control and 5Kx samples compared to the 20Kx samples (*, $p < 0.05$).

Figure 7.
Cryo-TEM image of liposomes functionalized with 1.6 mol% PR_b PA.

Figure 8.

Liposomes functionalized with PR_b PA or with no peptide were delivered to islets on the day of isolation (row 1 and 2) or day 2 of culture (row 3 and 4) and allowed to internalize for 24 (row 1 and 3) or 48 (row 2 and 4) hours. Confocal images show the cell membrane (red), nucleus (blue), and the liposomes (green). The images are 2D z-projections of the fluorescent features from the multiple layers of the islet that were captured. At least 2 different pig isolations were tested for each time point and images are shown from a single experiment. All scale bars are 20 μ m.

Figure 9.

Flow cytometry data of liposomes added to the culture on day 0 and allowed to incubate with the islets for 24 hours before analysis. The percentages of cells with a positive fluorescent shift compared to the control are shown in the inset table. A positive shift in fluorescence represents binding and/or internalization of calcein loaded liposomes into the islet cells. The results are representative for $n=2$. Results shown are from a single experiment and the other experiment is shown in the Supporting Information (Figure S3).