Scaffold Architecture Controls Insulinoma Clustering, Viability, and Insulin Production

Britani N. Blackstone, MS,¹ Andre F. Palmer, PhD,² Horacio R. Rilo, MD,³ and Heather M. Powell, PhD^{1,4}

Recently, *in vitro* diagnostic tools have shifted focus toward personalized medicine by incorporating patient cells into traditional test beds. These cell-based platforms commonly utilize two-dimensional substrates that lack the ability to support three-dimensional cell structures seen *in vivo*. As monolayer cell cultures have previously been shown to function differently than cells *in vivo*, the results of such *in vitro* tests may not accurately reflect cell response *in vivo*. It is therefore of interest to determine the relationships between substrate architecture, cell structure, and cell function in 3D cell-based platforms. To investigate the effect of substrate architecture on insulinoma organization and function, insulinomas were seeded onto 2D gelatin substrates and 3D fibrous gelatin scaffolds with three distinct fiber diameters and fiber densities. Cell viability and clustering was assessed at culture days 3, 5, and 7 with baseline insulin secretion and glucose-stimulated insulin production measured at day 7. Small, closely spaced gelatin fibers promoted the formation of large, rounded insulinoma clusters, whereas monolayer organization and large fibers prevented cell clustering and reduced glucose-stimulated insulin production. Taken together, these data show that scaffold properties can be used to control the organization and function of insulin-producing cells and may be useful as a 3D test bed for diabetes drug development.

Introduction

IN VITRO DIAGNOSTIC TOOLS are widely used in the clinical setting and traditionally include devices or kits that screen blood and other fluids for markers of disease, illness, or foreign substances. However, a new generation of *in vitro* diagnostic tools are being developed, which incorporate cells for more complex analyses such as predicting the biological response to drug therapies. These cell-based diagnostic platforms facilitate the screening of materials or drugs for toxicity or efficacy in an inexpensive, high-throughput manner. Additionally, such diagnostics allow for the down-selection of the most efficacious, safest treatments prior to testing on animal models. Unfortunately, most of these *in vitro* evaluations are performed using cells seeded onto standard tissue culture plastic (TCP)^{1–4} or biologically functionalized or coated TCP.^{5,6} Though 2D studies can provide useful information, these substrates lack physical homology with the native extracellular matrix, which can alter cell behavior and reduce the ability to translate laboratory findings to the clinic. As a result, new test beds for *in vitro* diagnostics must be developed, which promote 3D cellular architecture and increase homology to the native environment.

Traditional culture methods do not provide the complex 3D microenvironment that is necessary for many cell types to maintain or recreate their native, *in vivo* form and function. Many cell types, including hepatocytes, chondrocytes and β -cells, function in a more biomimetic manner when the cells are able to aggregate.^{7–11} For example, cell–cell interactions have been shown to influence β -cell function with cells in contact with each other secreting more insulin when stimulated than individual cells.^{12,13} Additionally, the relationship between the clustered structure of insulin-producing cells and their function has been well documented. Islets that retain their shape and β -cells and insulinomas that form aggregates exhibit increased insulin secretion compared with their monolayer forms.^{8–10,13} This increase in biomimetic function is proposed to be due to the increased cell–cell interactions^{12–14} that cells experience when included in an aggregate as opposed to in a monolayer. Many methods have been utilized to encourage cell clustering with the most effective of these relying on complex systems or nanofabrication techniques.^{15–18}

A potentially simpler solution is to control aggregation via scaffold morphology. Scaffold or substrate morphology has previously been shown to control individual cell

¹Department of Biomedical Engineering, The Ohio State University, Columbus, Ohio.

²William G. Lowrie Department of Chemical and Biomolecular Engineering, The Ohio State University, Columbus, Ohio.

³Department of Surgery, Arizona Diabetes Center, University of Arizona, Tucson, Arizona.

⁴Department of Materials Science and Engineering, The Ohio State University, Columbus, Ohio.

morphology and induction of organization into multicellular aggregates.^{19–24} Electrospinning provides an ideal platform for investigating the specific effects of scaffold morphology variation as fabrication parameters such as solution concentration, fluid flow rate, and applied voltage can be utilized to generate scaffolds with varying fiber diameter, fiber density, and porosity, without changing the raw material.^{25–28} Thus, the goal of this study was to investigate the effect of electrospun scaffold architecture on the organization and insulin production of insulinomas. Electrospun scaffolds were manufactured to possess three distinct fiber diameters and inter-fiber distances with two-dimensional substrates of the same material serving as a control. Insulinoma viability, cluster size and shape, and both baseline and glucose-stimulated insulin production were assessed as a function of scaffold type.

Materials and Methods

Gelatin scaffolds

Scaffolds were electrospun from a solution of gelatin derived from bovine skin (Type B; Sigma-Aldrich, St. Louis, MO) and 2,2,2-trifluoroethanol (TFE; Sigma-Aldrich) at 8, 12, and 16 wt./vol.%. Gelatin solutions were pumped through a syringe pump (KD Scientific, Holliston, MA) at a flow rate of 10 mL/h for the 8% solution and 12 mL/h for both the 12% and 16% solutions. A positive applied voltage between 22.5 and 25 kV was used for all solutions to generate nonwoven scaffolds of gelatin ~400 μ m thick. Gelatin-coated glass disks, fabricated by treating each glass disk with soluble gelatin attachment factor (Invitrogen, Portland, OR) for 30 min, were used as a two-dimensional control. Scaffolds were physically cross-linked using dehydrothermal treatment (DHT) at –80 kPa and 140°C for 24 h and subsequently, cross-linked in 7 mM *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC; Sigma-Aldrich) in pure ethanol for 24 h at room temperature to improve biostability. All substrates were prepared for insulinoma inoculations by disinfecting in 70% ethanol for 24 h, rinsing in phosphate-buffered saline solution (PBS; Sigma-Aldrich), followed by five rinses in HEPES-buffered saline, and one rinse in cell culture medium (RPMI-1640; Invitrogen).

Scanning electron microscopy

The structure of the scaffolds and gelatin-coated glass disks was characterized using scanning electron microscopy (Sirion; FEI, Hillsboro, OR). Dry scaffolds, post-DHT, were attached to aluminum (Al) stubs using adhesive carbon paint, sputter coated with gold-palladium and imaged in secondary electron mode at 5 kV. Using Image J software (NIH, Bethesda, MD), the average fiber diameter was quantified for each scaffold type. Average fiber diameter was calculated from measurements on 300 individual fibers and reported as the average fiber diameter $\pm$ standard error of the mean (SEM). The pore size of each scaffold was approximated by measuring the distance between two adjacent fibers within the same plane with at least 100 measurements per scaffold. The average interfiber distance $\pm$ SEM was reported.

Cell culture

Rat INS-1 insulinomas were maintained in RPMI-1640 with L-glutamine (11.11 mM glucose; Invitrogen) supple-

mented with 10% heat-inactivated fetal bovine serum (Invitrogen), 50 μ M 2-mercaptoethanol (Invitrogen), 1 mM MEM-sodium pyruvate (Invitrogen), and 10 mM HEPES (Invitrogen). Cells were cultured until ~60% confluent prior to harvesting. Cells were then inoculated onto gelatin scaffolds and gelatin-coated glass discs at a density of 200,000 cells/cm². Scaffolds and discs were cultured for 7 days with medium exchanged every third day and samples removed for analysis on days 3, 5, and 7.

Insulinoma viability and proliferation

Cellular metabolism on gelatin scaffolds and gelatin-coated glass discs was assessed using an 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. At days 3, 5, and 7, the 12-mm gelatin-coated glass discs and 12-mm biopsy punches from the gelatin scaffolds were removed from culture and placed in a sterile solution of 0.5 mg/mL MTT in PBS. Discs ($n=6$) were incubated in 0.5 mL of solution for 12 h at 37°C, 5% CO₂. The MTT solution was then removed and replaced with an equivalent amount of methoxyethanol and placed on a rocker plate for 3 h at room temperature. A microplate reader (SpectraMax 190; Molecular Devices, Sunnyvale, CA) was used to quantify the amount of formazan product formed with values reported as the mean optical density $\pm$ standard deviation.

Cell proliferation as a function of time and substrate was quantified using DNA analysis. Biopsy punches, 6-mm in diameter, were collected from each substrate condition at days 3, 5, and 7 ($n=6$ per condition) and snap frozen. DNA was extracted from the samples using a DNeasy[®] Blood & Tissue Kit (Qiagen, Germantown, MD) following manufacturer's instructions. Absorbance of the DNA yield was measured at 260 nm with a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and DNA concentration was calculated against a standard curve. To calculate total cell number, a standard curve for DNA concentration versus cell number was constructed and number of cells in each sample was extrapolated from the standard curve. Average cell number $\pm$ standard deviation was plotted.

Confocal microscopy

To quantify insulinoma clustering as a function of culture time and growth substrate, samples were removed from culture at days 3, 5, and 7 ($n=4$ per time point). Samples were then rinsed with PBS thrice for 5 min each, fixed in 4% paraformaldehyde in PBS for 1 h at room temperature, and again rinsed with PBS, twice for 5 min each. Fixed samples were stained with phalloidin (1:50, AlexaFluor phalloidin 594; Invitrogen) and DAPI (1:500; Invitrogen) and imaged with an Olympus FV1000-Spectral Confocal microscope (Olympus, Center Valley, PA) at 20 $\times$ magnification. Cell cluster diameter was measured on at least 50 cell clusters using ImageJ and the average cell cluster diameter $\pm$ SEM was reported. z-stacks of cell clusters on each substrate on day 7 were taken at 60 $\times$ magnification for qualitative analysis, with representative reconstructions shown.

Cellular morphology

The morphology of cell clusters on substrates was also evaluated using scanning electron microscopy as described

above. Fixed samples were rinsed with 0.1 M sucrose in PBS thrice for 5 min each followed by a graded ethanol series consisting of rinses in 50% and 70% EtOH for 5 min and 85%, 95%, and 100% (2×) for 10 min. Samples were then chemically dried using a graded hexamethyldisilazane (HMDS; Alfa Aesar, Ward Hill, MA) series of 3:1, 1:1, 1:3 (EtOH:HMDS), and pure HMDS for 1 h each, followed by an overnight soak in pure HMDS. Samples were mounted, coated, and imaged as described above.

Baseline and glucose-stimulated insulin production

Baseline cellular production of insulin and insulin production in response to a glucose challenge was quantified using a rat insulin enzyme-linked immunosorbent assay (ELISA) kit. On culture day 7, baseline insulin production was assessed by incubating eight samples from each substrate group with fresh RPMI (11.1 mM glucose) for 1 h. Medium from all samples was then collected and stored for insulin analysis. Subsequently, samples were starved by incubation with RPMI containing 2.8 mM glucose for 45 min and then stimulated with RPMI containing 28 mM glucose for 1 h. Medium and cells were immediately collected for DNA analysis, as described above, and for insulin quantification. Insulin present in the medium was determined using a rat insulin ELISA (Mercodia, Uppsala, Sweden). Samples were diluted 1:50 and run in duplicate. Absorbance of the assay was measured at 450 nm on a plate reader. The amount of insulin in fresh medium was also quantified and found to be 0.0 mg/mL for all fresh medium samples. Total DNA was quantified in each sample using an

Easy-DNA™ Kit (Invitrogen) as above. Glucose-stimulated insulin production was reported as an average fold change in insulin present after the 28 mM glucose challenge versus insulin present in baseline medium (11.1 mM glucose) on day 7.

Additional samples were collected prior to the high glucose challenge and after glucose stimulation (30 and 60 min following 28 mM glucose addition) for immunostaining. Samples ($n=4$ per time point) were fixed in 4% para-formaldehyde in PBS and immunostained with DAPI, C-peptide (1:700; Cell Signaling Technology, Danvers, MA), and insulin (1:500; Sigma-Aldrich). Insulin and C-peptide in insulinoma clusters were assessed at the top, middle, and bottom of each cluster using identical laser conditions for each antibody to compare relative abundance of each protein. Representative confocal sections from the midsection of the cell clusters were shown.

Statistical analysis

Statistical significance was determined by one-way analysis of variance (ANOVA) with a Tukey *post hoc* test. Significance was reported where $p < 0.05$.

Results

Gelatin scaffold morphology

Scanning electron microscopy of the substrates revealed that gelatin-coated glass discs were uniformly flat while the electrospun scaffolds consisted of randomly oriented ribbon-like fibers (Fig. 1). These fibers appeared to increase in fiber diameter and interfiber distance with increasing wt./vol.%.

FIG. 1. Scanning electron micrographs of the growth substrates and scaffolds. (A) Gelatin-coated glass disk and electrospun gelatin scaffolds at (B) 8, (C) 12, and (D) 16 wt./vol.%. Scale bar = 100 μ m.

FIG. 2. (A) Average fiber diameter in electrospun gelatin scaffolds as a function of solution concentration. (B) Average interfiber spacing in electrospun gelatin scaffolds as a function of solution concentration.

This observation was confirmed with image analysis (Fig. 2). Both fiber diameter and interfiber distance significantly increased with increasing solution concentration.

Cell viability and proliferation

Cellular metabolism, as measured by the MTT assay, showed significant differences as a function of substrate type (Fig. 3A). For all 3 days, cells on the gelatin-coated discs exhibited the lowest metabolism while insulinomas grown in the 16% gelatin scaffold had significantly increased metabolic activity. At all days, cell metabolism scaled positively with increasing scaffold fiber diameter and increased electrospinning solution concentration (Fig. 3A).

On culture days 3, 5, and 7, cell number was the lowest on the gelatin-coated disks, with all 3D conditions exhibiting significantly larger cell numbers (Fig. 3B). Cell number on days 5 and 7 increased on fibers of larger diameter and interfiber distance (Fig. 3B). As cell metabolism and number followed a similar trend, the increased metabolism on fibrous substrates was attributed to increased cell density.

Cell morphology

Insulinoma morphology was evaluated at time points between 3 and 7 days. On all substrates, insulinomas exhibited a clustered morphology with cluster size increasing with time in culture (Fig. 4). On gelatin-coated 2D substrates, insulinomas formed large, flat clusters within a few days of inoculation and by day 7, the entire substrate was covered by a few very large clusters (Fig. 4I). In contrast, insulinomas cultured in 3D fibrous scaffolds formed rounded clusters that were slower to form. A trend of decreasing insulinoma cluster size with increasing fiber diameter was apparent by day 7, with the thin gelatin fibers (8% gelatin) encouraging the formation of large insulinoma clusters (Fig. 4J–L). Insulinoma cluster location within the scaffold was also greatly affected by fiber diameter; small, thin fibers produced large clusters on top of the scaffold, whereas clusters formed deeper within the scaffold when it was comprised of thicker fibers (Fig. 4J, L).

FIG. 3. (A) MTT assay of INS-1 cell metabolism and (B) total cell number as a function of day and scaffold type.

FIG. 4. Scanning electron micrographs of INS-1 cells cultures in gelatin-coated glass (A, E, I), 8% gelatin scaffolds (B, F, J), 12% gelatin scaffolds (C, G, K), and 16% gelatin scaffolds (D, H, L) at 3 (A–D), 5 (E–H) and 7 (I–L) days in culture. Scale bar = 50 µm.

Confocal image analysis of insulinoma cluster area on the respective substrates supported the visual observations in the SEM. At day 3, insulinoma clusters were on average largest on gelatin-coated 2D substrates followed by 8% gelatin fibers (Fig. 5). At day 7, insulinoma cluster area was roughly fourfold larger than that of the 8% gelatin scaffolds. Insulinoma cluster size was observed to be inversely proportional to electrospon solution concentration and fiber diameter (Fig. 5). To evaluate the thickness and shape of the insulinoma clusters, confocal imaging was used to compile

FIG. 5. Insulinoma cluster diameter, calculated from single plane confocal images, as a function of culture time and scaffold type. Solid surfaces promote the formation of large INS-1 clusters while scaffolds with thicker, less dense fibers support small INS-1 clusters.

3D images of the cell clusters. Compiled confocal z-stacks showed that the insulinoma clusters on the 2D gelatin-coated substrates were typically one to two cells thick and at most three cell layers thick (Fig. 6A). Clusters on 8% gelatin scaffolds were round in shape and remained on the top plane of the scaffolds (Fig. 6B). In contrast, small 10–15 cell clusters were at multiple levels within the more porous 16% gelatin scaffold (Fig. 6D).

Glucose-stimulated insulin production

Insulin production in response to a glucose spike was improved when INS-1 cells were cultured on the 8% and 12% gelatin scaffolds compared with the 2D substrate and the 16% scaffold (Fig. 7). The fold increase in insulin production when cells on 8% or 12% gelatin scaffolds were exposed to 28 mM glucose-containing RPMI was over 1.5-fold compared with day 7 baseline values (medium containing 11.1 mM glucose) whereas glucose-stimulated insulin production was slightly over onefold in the 2D and 16% groups (Fig. 7). No insulin was detected in medium-only samples.

Immunostaining for C-peptide and insulin prior to and at 30 min intervals following glucose enrichment showed that C-peptide and insulin expression were present throughout the clusters formed on gelatin fibers and 2D substrates (Figs. 8–10). After incubation in 2.8 mM glucose, insulin and C-peptide staining was most intense on the periphery of clusters on the 2D substrates with diffuse staining in the 12% and 16% groups (Fig. 8). After 30 min at 28 mM glucose, both C-peptide and insulin staining was notably more intense than after glucose starving in the 8% and 12% groups (Fig. 9) with a reduction in C-peptide staining intensity observed in these groups at 60 min (Fig. 10).

FIG. 6. Confocal z-stack projections of INS-1 clusters on gelatin-coated glass disks (**A**) and within electrospun gelatin scaffolds (8%, **B**, 12%, **C**, and 16%, **D**). Translucent green areas mark the location of the scaffold or substrate. Color images available online at www.liebertpub.com/tea

Discussion

The two-dimensional substrates traditionally used for *in vitro* diagnostics cannot support the three-dimensional structures cells assume *in vivo*. Because cell structure has

FIG. 7. Fold change in insulin production by INS-1 cells after stimulation with 28 mM glucose for 1 h compared to baseline insulin production in normal medium (11.1 mM glucose) after 1 h.

been known to affect its functioning, results from such diagnostics may not translate to patient care. It is therefore necessary to develop a test bed that mimics the native microenvironment and can control cell structure and function. As scaffold or substrate morphology has been previously shown to control individual cell shape and organization in multicellular aggregates,^{19–24} it was hypothesized that scaffold morphology could control insulinoma clustering. Electrospinning was chosen for three-dimensional scaffold fabrication as its parameters can easily be controlled to vary fiber diameter and density and scaffold porosity.^{25–28} Gelatin was used as the substrate material as it is a fairly inexpensive natural protein that can easily be processed and controlled with electrospinning. Gelatin fiber diameter and interfiber distance scaled positively with solution concentration as previously demonstrated in the literature (Figs. 1 and 2).^{29,30}

In the current study, cell proliferation and metabolism were greatly affected by substrate architecture. Cell metabolism and number were lowest on the 2D gelatin-coated disks for all three time points. Proliferation and viability increased with fiber diameter and interfiber distance within the 3D electrospun scaffolds (Fig. 3). Improved cell viability and metabolism has been previously observed in other cell types when cultured on 2D versus 3D materials. Human mesenchymal stem cells cultured on type I collagen nanofibers for 7 days showed higher viability than those cultured on polystyrene.³¹ Over 9 days, human dermal fibroblasts exhibited higher cell counts on electrospun poly(methyl methacrylate) (PMMA) scaffolds than on films made of the same polymer.²² As 3D scaffolds provide significantly more surface area for cells to attach and proliferate²² contact inhibition likely does not play a role in the growth of cells on these scaffolds, whereas cells reach confluency quickly on 2D polymer films.

Confocal and scanning electron microscopy also showed that insulinomas on 2D gelatin quickly reached confluency and thus exhibited lower levels of cellular metabolism and proliferation due to contact inhibition. Insulinoma clusters on the gelatin-coated disks covered a significantly larger area than all other substrates on days 5 and 7 (Figs. 4 and 5). On the 2D substrates, clusters were no more than three cell layers thick with a large percentage of the surface covered in a monolayer. In contrast, 3D scaffolds promoted the formation of round cell clusters (Fig. 4). Electrospun scaffold structure had a clear impact on cell cluster morphology, as clusters decreased in size with increased fiber diameter and interfiber distance (Figs. 4 and 5). Clusters on the 8% gelatin scaffolds were the largest and formed on the surface of the scaffolds. In contrast, cells on 16% gelatin scaffolds formed more numerous, smaller clusters that were present deeper within the scaffolds (Figs. 4 and 6). As the interfiber distance in the 16% gelatin is the largest of the scaffolds used in this study, INS-1 cells inoculated into these scaffolds could have significantly increased initial cell penetration compared to the 8% or 12%. Fibroblasts inoculated onto gelatin scaffolds of varying fiber diameter were previously shown to penetrate to different initial depths based on fiber diameter with the greatest fiber diameter and interfiber distance promoting deeper infiltration.²⁹ The enhanced dispersion of cells throughout the 16% gelatin scaffold would then lead to greater distances cells must travel to cluster,

FIG. 8. C-peptide (green) and insulin (red) immunostaining of insulinoma clusters prior to glucose stimulation. Nuclei are stained with DAPI (blue). All images were taken from the midpoint of the insulinoma cluster. Scale bar = 50 μ m. Color images available online at www.liebertpub.com/tea

fewer cell–cell interactions and an overall smaller cluster size.

Though it is difficult to quantify given the difficult nature of live imaging within a fibrous scaffold, it is also possible that insulinoma migration on or within these scaffolds played a role in controlling the size and shape of the clusters. Scaffold/substrate morphology has been previously shown to control migration on constructs. Corneal and vascular cells from chick embryos showed increased migration area on rough PMMA than that of smooth PMMA.³² Nanofibrous scaffolds promoted greater migration of mouse fibroblasts than films composed of the same materials.³³ Human mesenchymal stem cells on poly(styrene) nanofibers also exhibited an increased migration area of 37.3%–56.7% compared to 2D polystyrene.³¹ As 8% gelatin scaffolds were comprised of densely packed thin fibers, the insulinomas may have perceived this scaffold to be similar to a roughened 2D substrate and as a result were able to migrate farther distances to form few larger, round clusters. As fiber diameter and “pore space” increased, insulinomas could more easily reach the inner scaffold during inoculation. This phenomenon has been previously observed in electrospun gelatin when human dermal fibroblasts penetrated 85% of the total scaffold thickness in a 16 wt./vol.% gelatin scaffolds, whereas penetration was limited to 61% of the scaffold thickness in a 10 wt./vol.% gelatin scaffold.²⁹ It is likely that insulinomas penetrated into the higher percentage (i.e., 16 wt./vol.%) scaffolds more readily, resulting in

decreased local cell density. As a result, their ability to form large clusters may be inhibited as the cells would be required to travel long distances to form large clusters.

Glucose-stimulated insulin production was also affected by cluster size and shape. Insulin production by insulinomas cultured on 8% and 12% gelatin scaffolds exhibited the largest fold increase over baseline compared with 2D and 16% scaffolds (Fig. 7). It appeared that larger rounded clusters maintained the INS-1 cells responsiveness to glucose stimulation, whereas monolayer-like and smaller rounded clusters led to a nonresponsive phenotype in these cells and released similar amounts of insulin per ng DNA regardless of glucose concentration. Previous experiments with islets and various substrates have exhibited a similar relationship between structure and function. Islet monolayers cultured on collagen gel formed pseudoislets and increased insulin secretion in comparison to those on TCP.⁹ Similarly, dissociated rat islets in collagen sponge-PLGA mesh composites formed rounded clusters and showed enhanced insulin production over monolayers of cells on tissue culture dishes.⁸ Suspensions of dissociated human islets in a rotational cell culture system formed clusters by day 3, with increased insulin secretion over intact islets that did not retain their rounded shape when cultured in traditional dishes.¹⁰ This trend of increased insulin production versus baseline in the 8% and 12% groups may be due to increased cell–cell interactions in these close-packed clusters. Cell–cell interactions are thought to be primarily responsible for

FIG. 9. C-peptide (green) and insulin (red) immunostaining of insulinoma clusters 30 min after stimulation with 28 mM glucose-containing RPMI. Nuclei are stained with DAPI (blue). All images were taken from the midpoint of the insulinoma cluster. Scale bar = 50 μ m. Color images available online at www.liebertpub.com/tea

FIG. 10. C-peptide (green) and insulin (red) immunostaining of insulinoma clusters 60 min after stimulation with 28 mM glucose-containing RPMI. Nuclei are stained with DAPI (blue). All images were taken from the midpoint of the insulinoma cluster. Scale bar = 50 μ m. Color images available online at www.liebertpub.com/tea

stimulating insulin response¹² and it has been proposed that a critical number of cell–cell contacts are required for coordinated insulin bursting with at least four β -cell to β -cell cell contacts needed.¹⁸ Additionally, reports have indicated that packing density of β -cells, not just total number of cells within a cluster, control burst ability and synchronization with larger, hexagonally close-packed clusters exhibiting the largest percentage of cells bursting in synchronization.³⁴ Similar results to the current study were recently observed in microprinted insulinoma clusters where glucose-stimulated (15 mM) insulin production over starved baseline (0.0 mM) was correlated with cluster size with normalized insulin production increasing in a relatively linear fashion from 40 up to 120 μ m clusters.¹⁸ As seen in the current study, small microprinted clusters produced equivalent or slightly less than onefold insulin production versus baseline.¹⁸

A significant increase in insulin production in response to glucose stimulation, over normal medium, was exhibited by 8% and 12% gelatin scaffold samples. A similar trend in immunostaining intensity for C-peptide and insulin was observed in these groups. C-peptide links the α and β chains of insulin, forming proinsulin, and aids in the assembly and processing of insulin.³⁵ Because positive insulin and C-peptide staining may individually reflect residual or exogenous protein presence, dual staining was used to indicate new protein formation.³⁶ C-peptide and insulin were seen throughout the entire volume of most clusters on gelatin fibers, however, they were most abundant in 8% and 12% gelatin scaffolds at 30 and 60 min post stimulation (Figs. 9 and 10). The results here support the recent findings that insulinoma aggregate diameters greater than 60 μ m (cells on 8% or 12% gelatin scaffolds) synthesize more *de novo* insulin per cell in response to sustained glucose stimulation and that cluster diameter affects the rapidity of insulin release.¹⁸

Limitations to the present study involve the ability to capture the cell clustering process and insulin production in real time. To definitively identify a root cause for differences in cell clustering among the scaffold groups, real-time imaging of cell migration and clustering is needed. Due to 3D nature of both the scaffold and the cell clusters, real-time imaging was extremely challenging and was not able to provide information from deeper than 10–20 μ m in the scaffold; thus, we were not able to watch the clustering process, only observed snapshots in time. Though we postulate that initial cell infiltration and differences in cell migration on highly porous versus tightly woven scaffolds controlled clustering ability, there is a possibility that other factors played a role. Additionally, the inability to capture high quality live imaging throughout the entire cell cluster during the glucose challenge and cell loss during DNA quantification adds an additional complication to the interpretation of the cell cluster responsiveness to the glucose spike. As cell clusters are not tightly adhered to the substrates, cell loss from scaffolds due to the rinsing procedure required for the DNA analysis may have led to an overestimation of insulin production versus ng DNA. Additionally, we anticipate that the more porous scaffolds assisted in “trapping” cell clusters within the scaffold causing their insulin production/ng DNA to be lower respectively. As all data were presented as fold change in insulin production versus baseline, we do not anticipate that these issues

would alter the trends observed. In future experiments, cell signaling and cell–cell communication will be directly probed to better understand why cluster size alters stimulation indices.

Conclusions

The results of this study show that substrate morphology controls insulinoma viability, cluster formation, and insulin response. Small, densely packed nanofiber scaffolds promoted the formation of large, round cell clusters and enhanced insulin production in response to glucose stimulation likely due to improved cell motility and enhanced cell–cell communication. The ability to control the 3D organization of cells and their function through scaffold manipulation will aid in the development of new diagnostic test beds for drug development and personalized medicine.

Acknowledgments

This article is based upon work supported in part by the National Science Foundation under Grant No. EEC-0914790 and by the Oak Ridge Associated Universities Ralph E. Powe Junior Faculty Enhancement Award (H.M.P.).

Disclosure Statement

No competing financial interests exist.

References

1. Chen, W.P., Chi, T.C., Chuang, L.M., and Su, M.J. Reversatrol enhances insulin secretion by blocking K_{ATP} and K_V channels of beta cells. *Eur J Pharmacol* **568**, 269, 2007.
2. Huotari, M.A., Palgi, J., and Otonkoski, T. Growth factor-mediated proliferation and differentiation of insulin-producing INS-1 and RINm5F cells: identification of betacellulin as a novel β -cell mitogen. *Endocrinology* **139**, 1494, 1998.
3. Moorwood, C., Lozynska, O., Suri, N., Napper, A.D., Diamond, S.L., and Khurana, T.S. Drug discovery for duchenne muscular dystrophy via utrophin promoter activation screening. *PLoS One* **6**, e26169, 2011.
4. Zhou, G., Gingras, M.C., Liu, S.H., Li, D., Li, Z., Catania, R.L., Stehling, K.M., Li, M., Paganelli, G., Gibbs, R.A., DeMayo, F.J., Fisher, W.E., and Brunicardi, C. The hypofunctional effect of P335L single nucleotide polymorphism on SSTR5 function. *World J Surg* **35**, 1715, 2011.
5. Atienzar, F.A., Tilmant, K., Gerets, H.H., Toussaint, G., Speckaert, S., Hanon, E., Depelchin, O., and Dhalluin, S. The use of real-time cell analyzer technology in drug discovery: defining optimal cell culture conditions and assay reproducibility with different adherent cellular models. *J Biomol Screen* **16**, 575, 2011.
6. Huet, C., Pisselet, C., Mandon-Pépin, B., Monget, P., and Monniaux, D. Extracellular matrix regulates ovine granulosa cell survival, proliferation and steroidogenesis: relationships between cell shape and function. *J Endocrinol* **169**, 347, 2001.
7. Abbott, J., and Holtzer, H. The loss of phenotypic traits by differentiated cells. 3. The reversible behavior of chondrocytes in primary cultures. *J Cell Biol* **28**, 473, 1966.
8. Kawazoe, N., Lin, X., Tateishi, T., and Chen, G. Three-dimensional cultures of rat pancreatic RIN-5F cells in

- porous PLGA-collagen hybrid scaffolds. *J Bioact Compat Polym* **24**, 25, 2009.
9. Lucas-Clerc, C., Massart, C., Campion, J.P., Launois, B., and Nicol, M. Long-term culture of human pancreatic islets in an extracellular matrix: morphological and metabolic effects. *Mol Cell Endocrinol* **94**, 9, 1993.
 10. Murray, H.E., Paget, M.B., and Downing, R. Preservation of glucose responsiveness in human islets maintained in a rotational cell culture system. *Mol Cell Endocrinol* **238**, 39, 2005.
 11. Shinji, T., Koide, N., Ogawa, A., Tanabe, T., Asano, K., Araki, Y., and Tsuji, T. Spheroidal aggregates of rat hepatocytes induced by rat liver biomatrix components in primary culture. *Jpn J Artif Organs* **17**, 179, 1988.
 12. Jacques, F., Jousset, H., Tomas, A., Prost, A.L., Wollheim, C.B., Irminger, J.C., Demaurex, N., and Halban, P.A. Dual effect of cell-cell contact disruption on cytosolic calcium and insulin secretion. *Endocrinology* **14**, 2494, 2008.
 13. Salomon, D., and Meda, P. Heterogeneity and contact-dependent regulation of hormone secretion by individual B cells. *Exp Cell Res* **162**, 507, 1986.
 14. Hauge-Evans, A.C., Squires, P.E., Persaud, S.J., and Jones, P.M. Pancreatic beta-cell-to-beta-cell interactions are required for integrated responses to nutrient stimuli: enhanced Ca^{2+} and insulin secretory responses of MIN6 pseudoislets. *Diabetes* **48**, 1402, 1999.
 15. Bueno, E.M., Bilgen, B., Carrier, R.L., and Barabino, G.A. Increased rate of chondrocyte aggregation in a wavy-walled bioreactor. *Biotechnol Bioeng* **88**, 767, 2004.
 16. Gallego-Perez, D., Higuera-Castro, N., Sharma, S., Reen, R.K., Palmer, A.F., Gooch, K.J., Lee, L.J., Lannutti, J.J., and Hansford, D.J. High throughput assembly of spatially controlled 3D cell clusters on a micro/nanoplatfrom. *Lab Chip* **10**, 775, 2010.
 17. Khaoustov, V.I., Darlington, G.J., Soriano, H.E., Krishnan, B., Risin, D., Pellis, N.R., and Yoffe, B. Induction of three-dimensional assembly of human liver cells by simulated microgravity. *In Vitro Cell Dev Biol* **35**, 501, 1999.
 18. Mendelsohn, A.D., Nyitray, C., Sena, M., and Desai, T.A. Size-controlled insulin-secreting cell clusters. *Acta Biomater* **8**, 4278, 2012.
 19. Andersson, A.S., Bäckhed, F., von Euler, A., Richter-Dahlfors, A., Sutherland, D., and Kasemo, B. Nanoscale features influence epithelial cell morphology and cytokine production. *Biomaterials* **24**, 3427, 2003.
 20. Brun, P., Abatangelo, G., Radice, M., Zocchi, V., Guidolin, D., Gordini, D.D., and Cortivo, R. Chondrocyte aggregation and reorganization into three-dimensional scaffolds. *J Biomed Mater Res* **46**, 337, 1999.
 21. Gerecht-Nir, S., Cohen, S., Ziskind, A., and Itskovitz-Eldor, J. Three-dimensional porous alginate scaffolds provide a conducive environment for generation of well-vascularized embryoid bodies from human embryonic stem cells. *Biotechnol Bioeng* **88**, 313, 2004.
 22. Liu, Y., Ji, Y., Ghosh, K., Clark, R.A.F., Huang, L., and Rafailovich, M.H. Effects of fiber orientation and diameter on the behavior of human dermal fibroblasts on electrospun PMMA scaffolds. *J Biomed Mater Res* **90A**, 1092, 2009.
 23. Moroni, L., Licht, R., de Boer, J., de Wijn, J.R., and van Blitterswijk, C.A. Fiber diameter and texture of electrospun PEOT/PBT scaffolds influence human mesenchymal stem cell proliferation and morphology, and the release of incorporated compounds. *Biomaterials* **27**, 4911, 2006.
 24. Poellman, M.J., Harrell, P.A., King, W.P., and Johnson, A.J.W. Geometric microenvironment directs cell morphology on topographically patterned hydrogel substrates. *Acta Biomater* **6**, 3514, 2010.
 25. Deitzel, J.M., Kleinmeyer, J., Harris, D., and Beck Tan, N.C. The effect of processing variables on the morphology of electrospun nanofibers and textiles. *Polymer* **42**, 261, 2000.
 26. Li, M., Mondrinos, M.J., Gandhi, M.R., Ko, F.K., Weiss, A.S., and Lelkes, P.I. Electrospun protein fibers as matrices for tissue engineering. *Biomaterials* **26**, 5999, 2005.
 27. McManus, M.C., Boland, E.D., Koo, H.P., Barnes, C.P., Pawlowski, K.J., Wnek, G.E., Simpson, D.G., and Bowlin, G.L. Mechanical properties of electrospun fibrinogen structures. *Acta Biomater* **2**, 19, 2005.
 28. Zong, X., Ran, S., Fang, D., Hsiao, B.S., and Chu, B. Control of structure, morphology and property in electrospun poly(glycolide-co-lactide) non-woven membranes via post-draw treatments. *Polymer* **17**, 4959, 2003.
 29. Powell, H.M., and Boyce, S.T. Fiber density of electrospun gelatin scaffolds regulates morphogenesis of dermal-epidermal skin substitutes. *J Biomed Mater Res A* **84A**, 1078, 2008.
 30. Zhang, Y., Ouyang, H., Lim, C.T., Ramakrishna, S., and Huang, Z.M. Electrospinning of gelatin fibers and gelatin/PCL composite fibrous scaffolds. *J Biomed Mater Res Part B* **72B**, 156, 2005.
 31. Shih, Y.R.V., Chen, C.N., Tsai, S.W., Wang, Y.J., and Lee, O.K. Growth of mesenchymal stem cells on electrospun type I collagen nanofibers. *Stem Cells* **24**, 2391, 2006.
 32. Lampin, M., Warocquier-Clerout, R., Legris, C., Degrange, M., and Sigot-Luizard, M.F. Correlation between substratum roughness and wettability, cell adhesion, and cell migration. *J Biomed Mater Res* **36**, 99, 1997.
 33. Meng, J., Kong, H., Han, Z., Wang, C., Zhu, G., Xie, S., and Xu, H. Enhancement of nanofibrous scaffold of multiwalled carbon nanotubes/polyurethane composite to the fibroblasts growth and biosynthesis. *J Biomed Mater Res* **88A**, 105, 2009.
 34. Nittala, A., Ghosh, S., and Wang, X. Investigating the role of islet cytoarchitecture in its oscillation using a new β -cell cluster model. *PLoS One* **10**, e983, 2007.
 35. Steiner, D.F., Cunnungham, D., Spigelman, L., and Aten, B. Insulin biosynthesis: evidence for a precursor. *Science* **157**, 697, 1967.
 36. Rajagopal, J., Anderson, W.J., Kume, S., Martinez, O.I., and Melton, D.A. Insulin staining of ES cell progeny from insulin uptake. *Science* **299**, 363, 2003.

Address correspondence to:
 Heather Megan Powell, PhD
 Department of Materials Science and Engineering
 The Ohio State University
 116 W. 19th Avenue
 243C Fontana Labs
 Columbus, OH 43210
 E-mail: powell.299@osu.edu

Received: February 11, 2013

Accepted: December 23, 2013

Online Publication Date: February 21, 2014