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RESEARCH PAPER

Integration of mesenchymal stem cells into islet cell spheroids improves long-term viability, but not islet function

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ABSTRACT

Pancreatic islets, especially the large islets (> 150μm in diameter) have poor survival rates in culture. Co-culturing with mesenchymal stem cells (MSCs) has been shown to improve islet survival and function. However, most co-culture studies have been comprised of MSC surrounding islets in the media. The purpose of this study was to determine whether islet survival and function was improved when the 2 populations of cells were intermingled with each other in a defined geometry. Hybrid spheroids containing 25, 50 or 75 or 90% islets cells with appropriate numbers of MSCs were created along with spheroids comprised of only islet cells or only MSCs. Spheroids were tested for yield, viability, diameter, cellular composition, and glucose-stimulated insulin secretion. The 25% islet/75% MSC group created the fewest spheroids, with the poorest survival and insulin secretion and the largest diameter. The remaining groups were highly viable with average diameters under 80μm at formation. However, the hybrid spheroid groups preferred to cluster in islet-only spheroids. The 50, 75 and 90% islet cell groups had excellent long-term survival with 90–95% viability at 2 weeks in culture, compared with the islet only group that were below 80% viability. The glucose-stimulated insulin secretion was not statistically different for the 50, 75, or 90 groups when exposed to 2.4, 16.8, or 22.4 mM glucose. Only the spheroids with 25% islet cells had a statistically lower levels of insulin release, and the 100% had statistically higher levels at 22.4 mM glucose and in response to secretagogue. Thus, imbedded co-culture improved long-term viability, but failed to enhance glucose-stimulated insulin secretion *in vitro*.

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Introduction

Islets of Langerhans, containing the insulin-producing cells of the body, are multicellular clusters of endocrine cells found in the pancreas that vary in size from 20 μm to more than 400 μm in diameter.[1] Pancreatic islets in the human body have a rich blood supply from a dense capillary network,[2] which is 10 times higher than that of the surrounding exocrine tissue. However, when these islets are isolated, the clustered cells must survive solely on diffusion.[3,4] Traditional cell culture approaches for islets often yield poor results with large size islets illustrating signs of core cell death with only 24 hours in culture.[5,6] For decades researchers have attempted to improve the culture conditions for isolated islets, with little effect on long-term outcomes.[7]

Co-culture is a popular procedure in which different populations of cells are cultured together either to investigate the cellular interaction between different cell types or to improve viability and function of the cells.[8] Certain cell types that are difficult to maintain in a monoculture, do better in a co-culture environment.[9-13] Cultured islets are already a co-culture system, as there are at least 4 primary endocrine cell types and several supporting cells in each islet. Loss of some of the cell types in the islet (namely endothelial and β cells) can partially account for reduced function of the islets when in culture.[14-16] Recently, the concept of adding extra support cells to the pancreatic islet culture has been investigated with several different cell types, with the goal of imiproving islet cell function and long-term viability by co-culturing non-islet cells into the culture media.

MSCs are a reasonable option for supporting islet health. MSCs can be derived from different adult

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tissues like bone marrow, adipose tissue and peripheral blood.[17,18] Adult MSCs have the ability to self-renew and self-proliferate.[19] MSCs help with the repair and regeneration of tissues of different origins like neuronal,[20] cardiac,[21] and integumentary.[22] Release of anti-apoptotic,[23] anti-inflammatory,[24,25] anti-oxidant,[26] immunosuppressive,[27,28] and angiogenic.[29-31] Specific to islet cells, MSCs appear to be protective, because when pancreatic islets were co-cultured with stem cells, their survival and function reportedly improved.[32-37]

Many techniques have been attempted to successfully co-culture islets with MSCs, including transwell plates, 3D scaffolds, and microfluidic platforms. In indirect co-culture, islets are separated from MSCs by a semi-permeable membrane. In direct co-culture, islets are allowed to directly contact the MSCs, either attached to a plate or in suspension.[38] A few studies have reported that direct co-culture is superior to indirect co-culture as defined by improvement in insulin secretion.[39] Direct contact with MSCs appears to help islets maintain their structural integrity.[40] Yet, even with direct contact, the islet cells remain separate, typically with MSCs attached to the outer layer.[38] If such islets were transplanted, it is likely that the exterior MSCs would be lost in the process. The goal of this study was to determine whether intermingling MSCs with islet cells in the same spheroid would further improve islet cell survival and

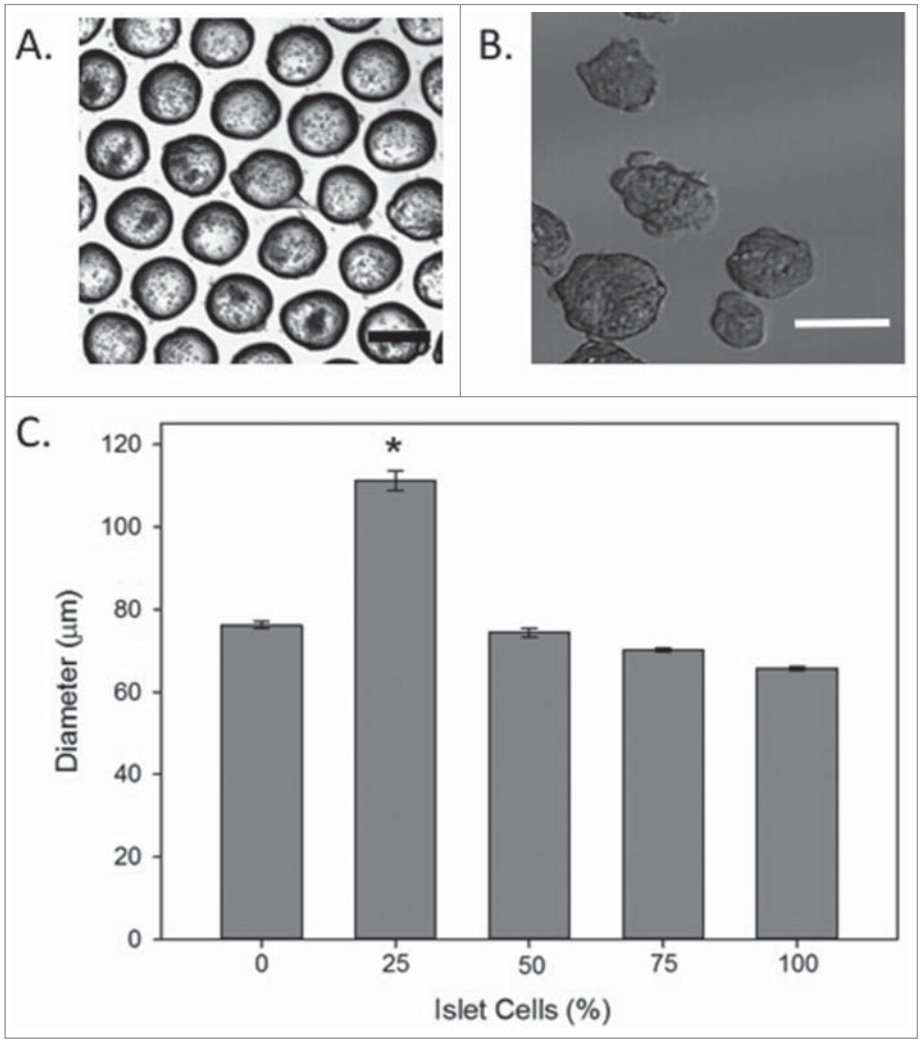

Figure 1. Formation of Spheroids. (A) Cells were loaded into micromold plates and began to cluster into spheroids within 24 hours. (B) By 3–5 days, mature spheroids were removed from the micromolds and used for testing. The image shows examples of pure MSC spheroids, termed 0% spheroids. Scale bar = 100μm for both images. (C) The average diameter of spheroids in each group at day 1 was below 100μm with the exception of the 25% islet group. For diameter measurements, spheroids from 4 independent trials were measured with a total of 135–285 spheroids/group. * indicates a significant increase in spheroid diameter, $p < 0.05$.

function, thus creating hybrid spheroids that could be transplanted.

Results

Formation of spheroids

Cells from all groups formed spheroids when cultured in the micromolds. Spheroids were formed within 3–5 d of co-culturing MSCs and islet cells, with pure MSC spheroids requiring the longest period of time for formation. Fig. 1 shows examples of MSCs loaded into the micromolds (Fig. 1A), and after removal from the molds (Fig. 1B). Interestingly, MSCs also appeared to spontaneously form spheroids without the micromold, although the sizes of spheroids varied widely (data not shown). The diameter of the spheroids was measured within 24 hours of removal from the micromold. The majority of spheroids were between 50–100μm in diameter (Fig. 1C). However, spheroids comprised of 25% islets/75% MSCs had the largest diameter (over 100μm), which was statistically greater than all other groups.

Yield of spheroids from different ratios of MSCs to islet cells

While approximately the same total number of cells were loaded into the molds to form spheroids in each group, the ability of the different cell mixtures to self-aggregate varied within and between the hybrid spheroid groups. The number of spheroids formed for the 25% islet cell group was approximately 500, which was approximately 4 times less than the group comprised of the 0, 50, 75, and 100% islet cells (Fig. 2). The within group variability is clear from the extent of the standard error bars. There was no significant difference in the yield of spheroids formed in any of the groups.

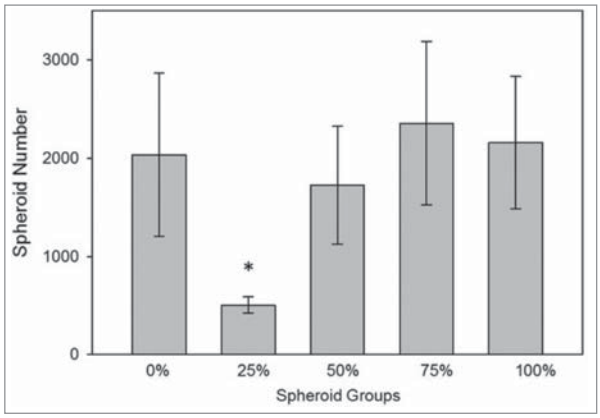

Figure 2. Spheroid Yield. The total number of spheroids formed per group were automatically counted on the day after removal from the micromold. Only the 25% group was statistically less than the other spheroid groups. Values = average of 3–4 independent trials.

Cell viability

The percentage of live cells was calculated from images of spheroids stained for apoptotic and necrotic cells (representative images shown in Fig. 3A-E). The images were collected within 24 hours of spheroid removal from the micromold with red indicating necrotic cell death and green/yellow indicating apoptotic cells. It is clear from the images that the 25% group had greater cell death than all other groups. In addition, the representative image shows an exceptionally large spheroid in the 25% group that was not uncommon as noted previously in summation of the diameter results. With the exception of the 25% group, all other groups had less than 10% dead cells. The cause of cell death (apoptosis versus necrosis) is summarized in Table 1. Most groups contained between 1–5% necrotic cells and 2–5% apoptotic cells, with the exception of the 25% group, which had a statistically higher percentage of apoptosis.

After 14 d in culture, there were not enough spheroids in the 25% group to evaluate. The 0, 50, 75, and 90% groups had between 2–5% apoptotic or necrotic cells after 14 d in culture. The exception was the 100% islet group, which contained over 80% dead cells with most of those staining positively for necrosis.

The low number of apoptotic or necrotic cells identified after 14 d in culture indicated healthy spheroids, and those groups containing MSCs continued to expand in number of the 2-week period. Table 2 summarizes the percent decline or increase in the number of spheroids per group. Only the 100% islet cell group, which contained no MSCs, declined in number during the 2-week period by nearly 30%.

Composition of hybrid spheroids

Describing the architecture of the hybrid spheroids was important, because we did not know if the cells would be randomly interspersed or bind only to like cells. Images of representative spheroids from some of the groups are shown in Fig. 4A with MSCs stained green and islet cells red. In general, more islet cell staining (red) was present as the ratio of islet cells to

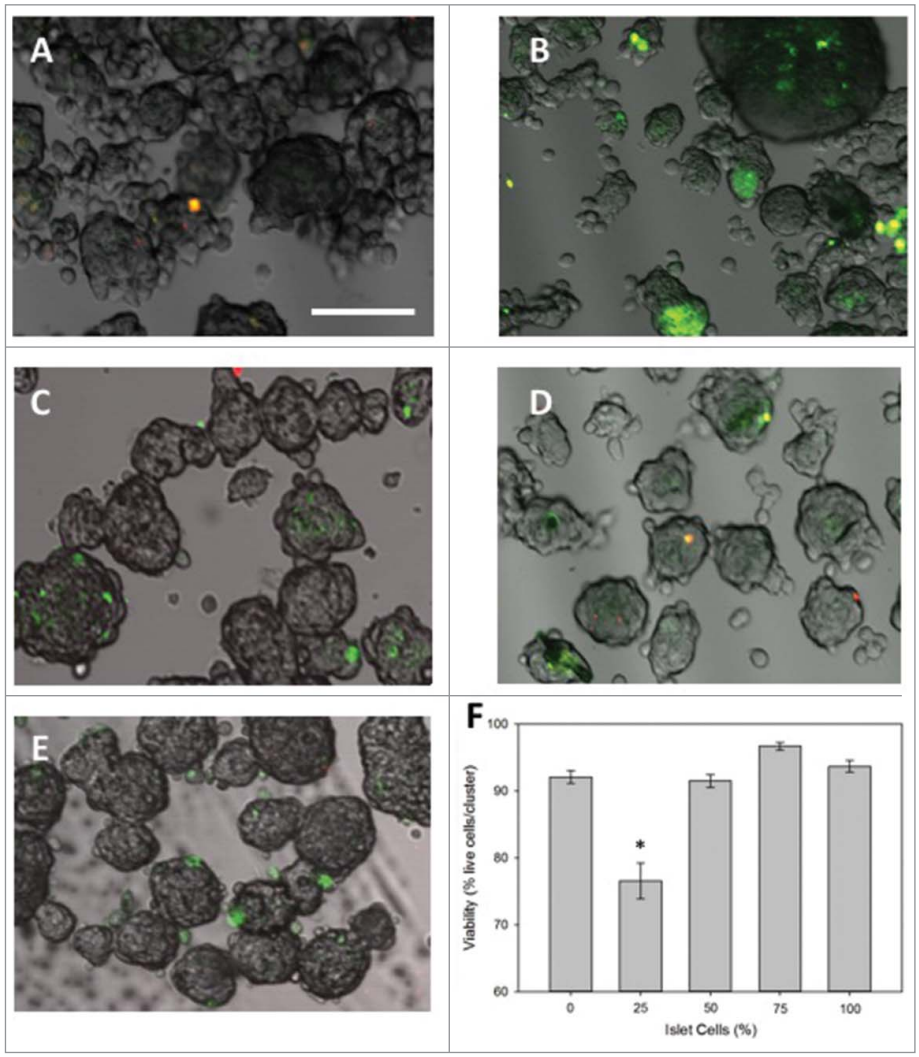

Figure 3. Cell viability. Representative examples of viability results are shown for (A) the 0% group, (B) 25%, (C) 50%, (D) 75%, and (E) 100%. Within 24 hours after removal from the micromolds, the 25% group had the highest percentage of apoptotic cells (green) with some necrotic cells (red or yellow). Scale bar = 100 μm applying to all images. (F) Cell viability was calculated for each group as the area without apoptotic or necrotic cells. The results are summarized in the bar graph. All the groups had high viability, although the 25% group was statistically lower than all other groups. Viability measurements were obtained from 790 – 1972 individual spheroids from 3–4 trials. * indicates $p < 0.05$.

MSCs increased from the 0% group to 50%, 75% and 100%. Within the hybrid groups, the MSCs and islet cells showed examples of random cellular distribution as shown in the example for the 75% group or co-localized together in regions found within the spheroids as shown in the 50% group example.

Table 1. Percentage of necrotic and apoptotic cells.

	1 Day		14 Days	
% Islet Cell Group	Necrosis	Apoptosis	Necrosis	Apoptosis
0%	5.20 ± 0.95	3.64 ± 0.41	2.94 ± 0.25	2.16 ± 0.27
25%	4.75 ± 0.72	18.69 ± 2.37 *	na	na
50%	4.22 ± 0.56	3.86 ± 0.43	2.93 ± 0.81	4.42 ± 0.61
75%	1.30 ± 0.22	1.96 ± 0.34	3.54 ± 0.39	4.19 ± 0.57
100%	3.20 ± 0.68	4.09 ± 0.56	13.71 ± 2.55 *	6.62 ± 1.45

To determine whether the MSCs localized to certain islet cells, additional staining was conducted with antibodies to insulin for β cells (green), glucagon for α cells (blue) and TGFβ receptor 1 (red), an MSC marker shown to be important in MSC renewal).[41] Fig. 4B shows an example of 3 spheroids made from the 50% loading protocol. The MSCs are not located on the periphery of the spheroids, but rather

Table 2. Survival of Spheroids over 14 day period.

Spheroid Group	% Change in Spheroid Number
0	35.72 ± 5.34
50	74.97 ± 4.35
75	37.99 ± 5.17
100	-29.00 + 5.32

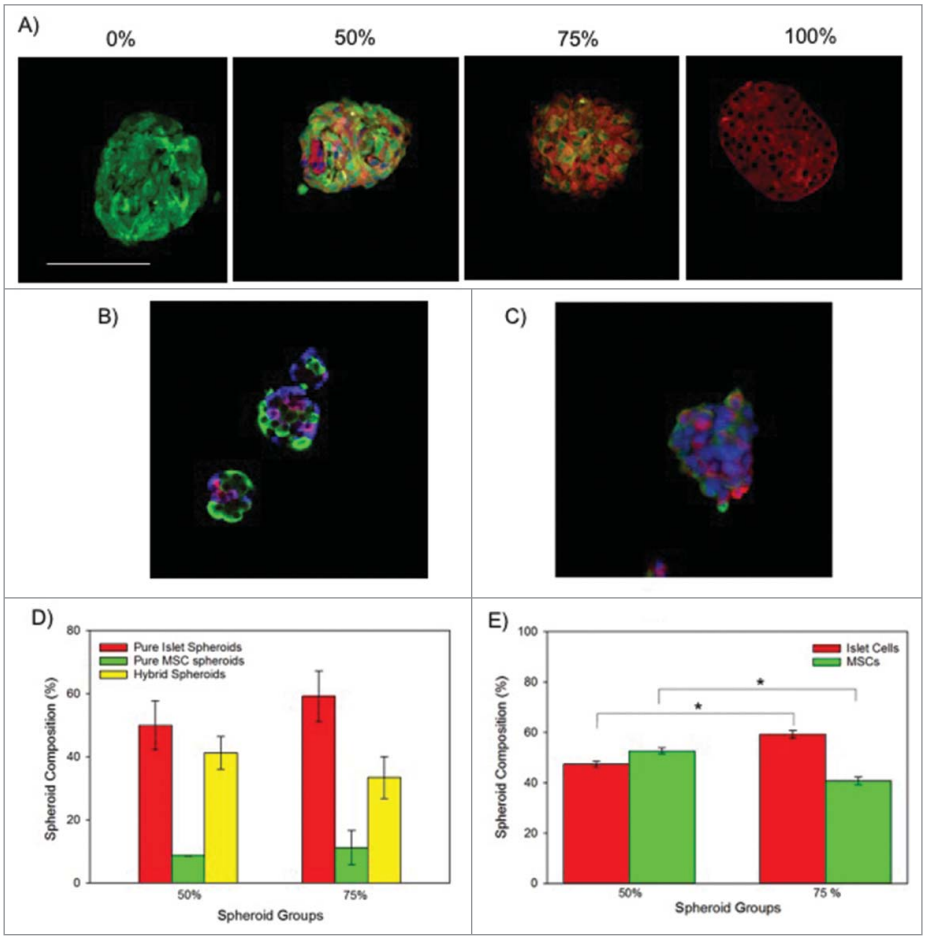

Figure 4. Spheroid Composition. (A) To track the co-localization of the groups of cells during reaggregation, MSCs were stained with CFDA-SE (green) and islet cells stained with DiI (red). Representative images of the resulting spheroids show the presence of the 2 different cell types when loaded with different proportions of islet cells. Scale bar = 100μm. (B) Examples of spheroids immunostained for β cells (anti-insulin, green), α cells (anti-glucagon, blue), and MSCs (anti-TGFβ receptor 1, red) are shown. (C) A 3D rendering of the immunofluorescence for β cells, α cells, and MSCs. (D) The proportion of spheroids with single cell type or multiple cell types (hybrids) shows that more islet cells aggregated together than mixed with MSCs. (E) Of the hybrids that had a mixture of MSCs and islet cells, the percentage of with within the hybrid followed the loading pattern. For D and E, 160–245 spheroids were analyzed per group. * indicates $p < 0.05$.

imbedded within the core. There was no obvious localization to β or α cells. Fig. 4C further illustrates the apparent random dispersion of the 3 different cells types in a 3D rendering of a spheroid.

Although cells in the hybrid groups were thoroughly mixed in given proportions before loading in the micromolds, spheroids were identified that contained only MSCs or only islet cells. The percentages of spheroids within a group consisting of a hybrid of islet cells and MSCs, vs. islet cells or MSCs alone were calculated (Fig. 4D). There was no significant difference in the percentages of spheroids consisting of only islet cells, only MSCs or both between the 50% and 75% hybrid groups, even though different proportions of islet cells and MSCs were loaded in the 2 groups. The majority of single-type spheroids within the hybrid groups consisted of islet cells only (Fig. 4D).

We completed additional analysis on the mixed cell-type hybrids. As shown in Fig. 4E, the proportion of stem cells or islet cells within the hybrid spheroids (containing both cell types) followed the loading proportions. There were more islet cells and fewer MSCs in the group loaded with 75% islet cells with respect to the group loaded with 50% islet cells.

Insulin secretion

The function of the islet cells 24 hours after removal from the micromolds was measured by challenging the spheroids to different concentrations of glucose

and measuring the secreted insulin. The lowest concentration tested was 2.4 mM glucose. Not surprisingly, the spheroids with only 25% islet failed to secrete as much insulin as the other groups at each glucose concentration (Fig. 5). There were no statistical differences between the 50, 75, 90 and 100% groups at 2.4 and 16.8 mM glucose exposures. At 22.4 mM glucose, only the 100% islet group had a statistically higher release of insulin compared with the other groups. When exposed to the secretegogue plus 22.4 mM glucose, the 90 and 100% groups showed statistical increases.

One hypothesis for the diminished insulin production was that the MSCs imbedded within the spheroids were interfering with the necessary cell-to-cell interaction required for normal insulin secretion. To test this hypothesis, another spheroid group was formed and tested comprised of 90% islet cells and 10% MSCs. The 90% spheroids had qualities similar to the other groups. Their average diameter was 76.15 ± 12.10 μm, statistically the same as the other groups, with the exception of the 25% group. Viability of the 90% group was 93% with 3.16% necrotic and 4.78% apoptotic cells. Surprisingly, 90% spheroids also failed to illustrate glucose sensitivity. This group of spheroids only showed a statistically significant increase in insulin release in response to a secretague, but not to high glucose concentrations (Fig. 5).

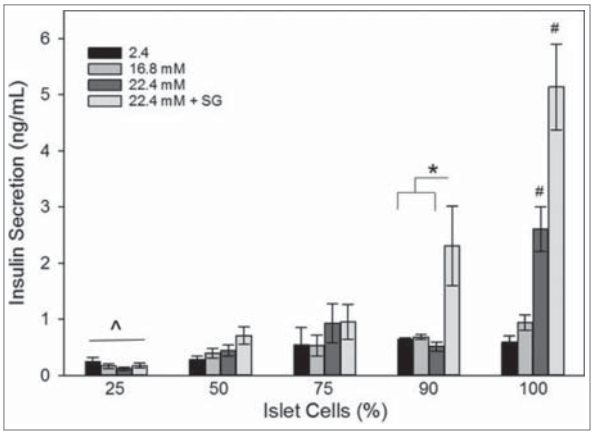

Figure 5. Glucose-Stimulated Insulin Secretion. Static insulin secretion was measured from spheroids over a 60 minute period. All groups released small levels of insulin in response to the low glucose concentration of 2.4 mM. As the glucose concentration increased, there was little increase in insulin secretion with the exception of the 100% pure islet cell group. (N = 4 trials with 6 replicates, with the exception of the 90% group) *indicates secretagogue + high glucose was different from within tests and between groups. # indicates within and between group differences. ^ indicates differences between groups at each glucose concentration. $p < 0.05$.

Discussion

Previous studies have used a variety of techniques to co-culture islet cells either directly or indirectly with MSCs. Rather than indirect co-culture using a transwell system,[35,37] or direct co-cultures,[39,40] the current study attempted to co-culture different proportions of islet cells and MSCs by incorporating them into the same spheroids, which may have led to a simpler method for transplantation. Islet cells were seeded on micromolds along with MSCs in different proportions and allowed to self-aggregate. We found that islet cells preferred to reaggregate in spheroids with other islet cells rather than in mixed hybrid spheroids with MSCs. This may be due to differences in the mode of cellular adhesion of pancreatic islets and MSCs. The cadherin superfamily largely regulates the aggregation and organization of pancreatic islet cells whereas MSCs are regulated by cell adhesion molecules of the immunoglobulin family.[42,43]

When there was a larger percentage of islet cells loaded into the micromold, it was easier for islet cells to reaggregate with each other. We are not the first to report such findings, as other researcher have demonstrated the challenge in creating MSC and islet hybrid clusters. Hoffecker and Iwata reported that without manipulating the cellular interaction to improve cohesiveness between MSCs and islet cells, MSCs and islet cells preferred to reaggregate with their own cell types.[44] In their study, MSCs were manipulated to express cadherins on their surface to improve contact between the other cell types.[44] In our study we found that, without manipulation of cell adhesion molecules, approximately 35–45% of the spheroids formed contained both cell types as shown in Fig. 3.

The results of our study demonstrate that it is possible to create highly viable hybrid spheroids consisting of MSCs and islet cells. With the exception of the 25% group, spheroids containing different proportions of MSCs and islet cells maintained high viability even after 14 d in culture, while islet-alone clusters had statistically greater cell death and loss of spheroids over time. These results are in agreement with the previous studies, which have shown that islet co-culture with MSCs improved the islet cell viability and survival.[33,36,45] Other studies have reported that the

viability of islets in direct co-culture was higher than islets in indirect co-culture or islets alone, however in the published direct co-culture study, viability was approximately 60% at 2 weeks,[39] whereas our spheroids were 90–95% viable at 2 weeks, compared with less than 80% viability in the islet only group.

The hybrid spheroids groups in this study failed to demonstrate enhanced insulin secretion in response to high glucose challenges (16.8 and 22.4 mM glucose). However, the decreased number of islet cells per spheroid, especially in the 25 and 50% groups, could have explained the results shown in Fig. 5. Therefore, additional calculations were completed adjusting the secreted insulin level to the number of islet cells loaded into the micromold (results not shown). Even with that additional normalization to islet cell loading, there was no enhancement of insulin secretion in the response to 16.8 or 22.4 mM glucose for any of the groups compared with the 100% islet cells. It is important to note that insulin secretion was measured 24 hour after removal of the clusters from the micromold, which equated to 4–5 d in culture. This time in culture may explain the damped GSIS results in all groups as has been suggested by others.[46]

The insulin secretion results were especially surprising, given that a large percentage of the spheroids in the hybrid groups were comprised of islet cells only, basically replicating an indirect co-culture model. The results are in contrast to previous studies that supported the concept that MSCs improved insulin secretion of islets.[40,45] Namely, Park et al. showed that when islets were in indirect co-culture with MSCs, insulin secretion was improved by nearly twice that of islet cells alone.[45] In a different study, islets co-cultured with MSCs indirectly had no significant difference in SI when compared with islets alone, whereas islets directly co-cultured with MSCs had SI values twice those of islets cultured alone at 3 weeks.[39]

In contrast to these positive results, we and others report a lack of enhanced glucose sensitivity with co-cultured MSCs. When MSCs were directly attached to islets, there appeared to be no benefit to insulin secretion. In one study, islets and MSCs were incubated for 3 hours in suspension resulting in adherence of the MSCs to the exterior of the islets. In this condition, the glucose sensitivity of the islet-MSCs spheroids was not significantly different than islets alone.[47] Another group found the same result when human islets were coated with MSCs and endothelial cells.[48] Finally, spheroids formed by manipulating the cellular interaction to improve cohesion between islet cells and MSCs, did not show an increase in insulin secretion with glucose challenges.[44] In our own results, there was no significant difference in the amount of insulin secreted in response to 16.8mM or 22.4mM glucose between the 50 and 75% groups. In fact, in response to 22.4 mM glucose, there was a statistically significant reduction in insulin secretion in all of the hybrid models compared with the islet cell group.

A major difference in our approach compared with the previous co-culture publications lies in our method for incorporating the MSCs into the islet spheroids in a way that altered the normal islet-to-islet cell interaction. While such a configuration dramatically improved islet viability, it failed to improve insulin secretion. It is quite possible that the 3D cell-to-cell interaction in an islet cell spheroid is essential to normal glucose sensing and insulin secretion. This theory is supported by different studies emphasizing the importance of cell-to-cell interactions between β cells for the normal function of islets.[49-52] Islet cells dispersed into single cells and attached to different substrates had stimulation indices of 1.2 – 2.4, in 3.3 vs. 20 mM glucose while in our study the 100% islet cells had a stimulation index of 5.2 in 2.4 and 22 mM glucose. Thus, our reaggregated 100% islet cells had a high stimulation index compared with other dispersed-cell models.[53] To test this hypothesis, we added a 90% islet cell group. While viability and survival in culture were extremely high in the 90% group, insulin secretion was still less than the 100% islet spheroids and was statistically less than the 100% group. Thus, what might be considered a minimal disruption of islet structure and cell interaction, still failed to improve function and actually diminished the amount of secreted insulin.

In summary, there is wide consensus that co-culture of stem cells with islet cells enhances cell viability and long-term culture *in vitro*. However, the effects of stem cells on islet cell function is still undetermined with approximately equal numbers of studies reporting positive results as those reporting no effect. This is important as several laboratories are working on improved islet health for transplantation, focusing on co-culture of the cells.[35,37-40,45]

In the current study, intermingling of the cells in 3D spheroids showed no improvement in glucose

sensitivity and some reduction of insulin secretion in response to high glucose challenges, but did enhance viability in long-term culture.

Methods

Isolation of rat pancreatic islets

Pancreatic islets were isolated and dispersed according to our published protocol.[3,5,54] The protocol for isolation of rat pancreatic islets was approved by Institutional Animal Care and Use Committee at University of Kansas Medical Center, USA. In brief, 38 Sprague Dawley rats (Harlan) were anesthetized by intraperitoneal injection of ketamine and xylazine. After opening the peritoneal cavity, the pancreatic main duct to the duodenum was clamped and distended with cold collagenase (CLS1, 450 units/mL; Cat. No. LS004197 Worthington). The pancreata were excised and incubated with gentle rotation for 20–30 min in a 37°C incubator. After washing and straining the contents of the tube through a 100-mm mesh screen, the pellet was mixed with Histopaque 1077 and 1119 (density = 1.1085; Cat. No. 11191 and 10771; Sigma Aldrich) and centrifuged. The islets, collected from the gradient, were sedimented and washed over a sterile 40-mm mesh cell strainer. Islets were placed into Connaught Medical Research Laboratories (CMRL; Cat. No. MT5110CV; Fisher Scientific) medium supplemented with 10% fetal bovine serum (FBS; Cat. No. 26140079; Life Technologies), 1% antibiotic/antimycotic (Cat. No. 15240062; Thermo Fisher Scientific) and 1% Glutamax (Cat. No. 35050061; Thermo Fisher Scientific) and allowed to recover for 24 hours in an incubator at 37°C and 5% CO_2.

Dispersing islets into single cells

Native rat islets were dispersed into single cells according to our previously published protocol.[55] In short, islets were collected in 50 ml tubes and centrifuged. Then the islets were washed twice with calcium- and magnesium-free Hank's Balanced Salt Solution (cmf-HBSS; Cat. No. SH30031.02; GE Healthcare Lifesciences). After that, the digestion medium, consisting of cmf-HBSS supplemented and papain (3 units/ml; Cat. No. 9001–73–4; Worthington), was added to the islets and the suspension was incubated on a rotator at 37°C for a maximum of 20 minutes. Islets were further dispersed using a pipette until the media primarily contained single cells. Digestion was stopped using CMRL medium containing calcium and magnesium. The cells were subsequently washed to remove the papain. Cells were brought up in CMRL medium containing 10% FBS, 1% antibiotic/antimycotic and 1% glutamax before mixing them with MSCs in appropriate proportion. Cell counts were performed using the EVE automated cell counter from NanEnTek (Cat No. 10027–452, VWR).

Creating MSC, islet and hybrid spheroids

Rat bone marrow MSCs (Cat No. RASMX-01001; Cyagen Biosciences Inc.) were thawed and expanded according to manufacturer's instructions. They were verified as MSCs by immunofluorescence described below. Once the appropriate number of cells were obtained, MSCs were trypsinized and dispersed in MSC growth media before mixing them with islet cells in defined proportions.

After performing the cell count for islet cells and MSCs, single cells were mixed in appropriate proportions and seeded into a micromold as described in our previously published protocol.[55] The cells were plated in the micromold with 3ml of culture media, incubated at 37°C and 5% CO_2. Cells re-aggregated and formed hybrid spheroids within 3–5 days, with daily media changes. Subsequently, they were removed from the mold by washing multiple times with the culture medium. Groups were identified according to the % of islet cells loaded onto the micromold with the 0% group representing pure MSC spheroids. Spheroids were counted at the time of removal from the micromold and 14 d later using the imaging capabilities of the BioTek Citation 5 Imaging Reader using the Gen 5 software, version 2.09.

Spheroid viability assessment

Viability was determined by double-labeling cells with Yo-Pro-1 and propidium iodide nucleic acid stains (Cat. No. V13243; Thermo Fisher Scientific). Yo-Pro-1 stain selectively passes through the plasma membranes of apoptotic cells and labels them with green fluorescence. Necrotic cells stained red-fluorescent with propidium iodide.[56] Yo-Pro-1 and propidium iodide (1μl of each) were added to 200μl of culture media containing spheroids from different groups. Images were taken using an Olympus Fluo View 300 confocal microscope, later the images were

analyzed using Abode Photoshop CS 5 extended. 30–150 images were analyzed per group. Total pixels, as well as the red and green pixels were calculated. The percentages of necrotic and apoptotic cells were calculated by dividing the pixels for red/ green staining to the total pixels and multiplying by 100.

Glucose-stimulated insulin secretion

Twenty-four hours after removal from the micromolds, spheroids were distributed in 96 well plates containing 100 μl of Earle's balanced salt solution (EBSS) with 2.8mM glucose. Spheroids from different groups were exposed to glucose concentrations of 2.8mM, 16.8 and 22.4mM for an hour in replicates of 6 following our previously published protocol.[54] After 60 min of static incubation at 37°C and 5% CO_2, conditioned media samples were collected and frozen at -80°C. The insulin concentration was later quantified using a rat insulin enzyme-linked immunosorbent assay kit (ELISA; Cat No. 80-INSRTH-E01; Alpco) as per the manufacturer's instructions. In short, standards and samples were added to the insulin specific monoclonal antibody coated microplate wells. After incubating the samples and standards with the detection antibody for 2 hours, the plates were washed with buffer. The wells were again incubated for 15 minutes after adding the substrate. Optical density was measured at 450nm by a spectrophotometer after adding the stop solution. Optical density values were divided by the number of islet cells in each well for normalization. SI was calculated by insulin secretion at high glucose exposure/islet cell to insulin secretion at low glucose exposure/islet cell.

Spheroid composition

To track the interspersion of the islet cells and MSC in the spheroids, the different cell types were labeled with distinct markers. Cells from pancreatic islets were labeled with DiI (1, 1′-dioctadecyl-3, 3, 3′, 3′-tetramethylindocarbocyanine perchlorate; Cat No. 42364, Sigma- Aldrich) and MSCs were labeled with CFDA-SE (carboxyfluorescein diacetate, succinimidyl ester; Cat. No. V12883; Thermo Fisher Scientific) according to the manufacturer's instructions. After centrifugation and aspiration of the supernatant, the MSC pellet was re-suspended in a pre-warmed (37°C) phosphate buffered saline containing 10μM of CFDA-SE. After a 15 minute incubation, the supernatant was removed following centrifugation at 1500 rpm for 5 minutes, and cells were re-suspended in pre-warmed fresh MSC medium with 10% FBS, 1% penicillin/ streptomycin and 1% glutamine for 30 minutes, pelleted and re-suspended in MSC medium with 10% FBS, 1% penicillin/ streptomycin and 1% glutamine. To stain the pancreatic islet cells with DiI, cells were re-suspended in 1 ml of serum free culture media with 5mM DiI and incubated for 15 minutes at 37°C. Cells were then centrifuged at 2500 rpm for 5 minutes and re-suspended in CMRL supplemented with 10% FBS, 1% antibiotic/ antimycotic and 1% glutamax.

After labeling, the islet cells and MSCs were mixed thoroughly in different proportions and were seeded into the micromold to allow them to reaggregate and form hybrid spheroids as described previously. Upon removal from the mold, the spheroids were placed on microscopic slides (Cat. No. 12–550–12; Fisher Scientific) and mounted with anti-fading agent gel/mount. Images were captured using a Nikon C1Si or C1 Plus confocal microscope. Interspersion data could only be obtained from spheroids less than 4 d after formation, because the fluorophores are depleted after that time. Images were analyzed using ImageJ to determine the percentage of islet cells and MSCs in each hybrid spheroid. In addition, analysis was completed to determine how many spheroids were comprised of a combination of islet cells and MSCs and how many consisted only of MSCs or islet cells alone.

The diameter of each spheroid was measured using Adobe Photoshop CS 5 from light microscopic images. Two perpendicular diameter measurements were recorded and averaged for each spheroid. 350- 2000 spheroids were analyzed for each group at days 1 and 14.

Immunofluorescence analysis

Rat MSCs were validated using antibodies to CD73 (cat no ab175396, Abcam), CD105 (cat no ab2529, Abcam), and CD34 (cat no ab213058), and TGFβ receptor 1 (ab31013, Abcam). The MSC cultured stained positive for CD73, CD105 and TGFβ receptor 1, but negative for CD34, validating the cell line as MSC.[57]

Mature spheroids were fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) using methods already published.[55] For cellular composition identification the

following antibodies were used: anti-insulin (1:100, cat no. ab7842, Abcam), anti-glucagon (1:200, cat no. ab53165, Abcam), and TGFβ receptor 1 (ab31013, Abcam). Corresponding secondary antibody conjugates included: Cy2 (Jackson ImmunoResearch Laboratories), Alexa 647 and Alexa 555 (Molecular Probes, Invitrogen).

Images were obtained using a Nikon C1Si or a C1Plus confocal microscope. Images were acquired at 10X to 100X and analyzed using Adobe Photoshop software. Renderings in 3D were accomplished using the Nikon C1Plus software.

Statistics

SigmaPlot software was used for data analysis. To determine significant differences between groups for the number of spheroids, proportion of spheroid type, proportion of islet cells and MSCs, viability, diameter, and insulin secretion one-way ANOVA was performed. To determine the significance within group differences at day 1 and day 14 for yield, spheroid survival, and viability, a matched t-test was performed. An α level of 0.05 was used to determine the significance of all findings.

Abbreviations

CFDA-SE	carboxyfluorescein diacetate, succinimidyl ester
Cmf-HBSS	calcium- and magnesium-free Hank's Balanced Salt Solution.
CMRL	Connaught Medical Research Laboratories
DiI	1, 1′-dioctadecyl-3, 3, 3′, 3′-tetramethylindocarbocyanine perchlorate
EBSS	Earle's balanced salt solution
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
MSCs	mesenchymal stem cells
SI	glucose stimulation index

Disclosure of potential conflicts of interest

S. R., K. R., and L. S. B. are inventors on patents using some of the procedures described here.

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