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ORIGINAL ARTICLE

Human BM stem cells initiate angiogenesis in human islets *in vitro*

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BM stem cells may have regenerative effects on islet function through angiogenesis. Human islets (100 islet equivalent/mL) were cultured alone (control) or co-cultured (experimental group) with whole human BM (1×10^6 cells/mL) for 210 days. A protein array measuring angiogenesis factors found upregulated (experimental vs control, day 210) proteins levels of VEGF-a (535 vs 2 pg/mL), PDGF (280.79 vs 0 pg/mL), KGF (939 vs 8 pg/mL), TIMP-1 (4592 vs 4332 pg/mL) and angiogenin (506 vs 97 pg/mL). Lower protein levels of angiopoietin-2 (5 vs 709 pg/mL) were observed. Depletion of pro-angiogenesis factors in co-culture decreased the effects of BM-induced islet vascularization. Depletion of VEGF-a, eKGF and PDGF significantly reduced islet vascular-ization but individual depletion of KGF and PDGF had less effects overall on vessel formation. BM-induced vascularization showed significant endothelial cell distri-bution. Islet vascularization was linked to islet growth. A decrease in islet size indicated poor vascularization. Insulin release was evident in the tissues generated from human islet-BM co-culture throughout the entire culture period. Significant increase in insulin (28.66-fold vs control) and glucagon (24.4-fold vs control) gene expres-sion suggest BM can induce endocrine cell regeneration. In conclusion, BM promotes human islet tissue regenera-tion via regulation of angiogenesis factors.
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Introduction

The significant loss of pancreatic islet function within a short period of time after transplantation greatly limits the efficacy of islet transplantation for diabetic patients. One of the major causes of islet damage may be due to the islet isolation process, which destroys the supportive microenvironment critical for islet survival. Many efforts have been made to develop methods and materials to preserve islet function during isolation.[1–3] Several studies have examined cellular signaling transduction pathways,[4] inflammatory factors[5,6] and vascularization of transplanted material[7] with the ultimate goal of improving clinical response. Recent evidence supports the ability of BM cells to repair non-hematopoietic tissues, including tissues in the central nervous system, kidney, lung and skin.[8–10] In this study, human BM was used to generate a suitable microenvironment to enhance longevity of human islets.[11] This may be a valuable approach for long-term islet survival and function, leading to successful islet transplan-tation.

Islet transplantation has been regarded as a potential cure of type I diabetes via the replacement of lost islets throughout the pathology of the disease. Two of the major roadblocks to islet transplantation have been the avail-ability of islet donors as well as maintaining islet function after isolation.[1–3] The current model for islet transplanta-tion has been the Edmonton protocol, which requires at least two donors per transplant.[11,12] Islets post-isolation have a poor survival rate possibly because of damage incurred throughout the isolation process.[11]

BM potentially facilitates human islet longevity through regenerating islet vascularization destroyed during the isolation process. It has been shown that the native pancreas has more than one and a half times the vessel density and oxygen tension of transplanted islets, suggest-ing that the lag in revascularization and the inferior vascular supply contributes to impaired islet function.[13]

Recently, we studied the use of BM-derived progenitor cells to support and regenerate new β-cells *in vitro*.[11] We found that multiple mechanisms may be involved in stimulating human islet regeneration—one of which is islet angiogenesis. Islet angiogenesis has been recognized to have a critical role in sustaining islet function and viability not only after human islet isolation *in vitro* but also for islet transplantation *in vivo*. We believe that BM initiates human islet revascularization resulting in the repair of human islet injury in long-term co-culture (over 2 years).[11]

BM-derived MSCs and endothelial progenitor cells naturally produce a variety of cytokines and growth factors to promote the survival of surrounding cells via paracrine mechanisms.[14,15] Angiogenesis factors released by BM may have a critical role in repairing islet injury.[16] In this study, we used a specific angiogenesis protein array to explore

whether there are factors released from BM during co-culture and whether these angiogenesis factors contribute to islet angiogenesis.[17] Using the protein array assay and specific Abs to deplete angiogenesis factors, we were able to identify whether angiogenesis factors released by BM have a role in human islet survival.

Materials and methods

Human pancreatic islets
Human islet tissue, from normal donors, was obtained from ICR (Islet Resource Centers) in the ICR Basic Science Islet Distribution Program, Human Islet Laboratory, University of Pennsylvania (Philadelphia, PA, USA), Massachusetts General Hospital (Boston, MA, USA) and City of Hope National Medical Center (Duarte, CA, USA). The use of these cells was approved by the IRB (Institutional Review Board) at Roger Williams Medical Center and the ICR Committees. Human islets were received within 48 h after harvest from cadaveric donors. The purity of islets in the total isolated tissue was 75–90% as assessed by dithizone identification and viability was >95% as determined by Trypan blue dye exclusion. Islets were placed in culture at 100 islet equivalents per mL. Cultures were maintained in RPMI 1640 (GIBCO, Invitrogen, Carlsbad, CA, USA) supplemented with 10% heated inactivated FBS (GIBCO), 5.5 mM glucose, 10 mM HEPES and 1% P/S.

Human BM
Human BM from normal donors was obtained under a separate Roger Williams Hospital IRB approved protocol. BM mononuclear cells were isolated by Ficoll-Paque Plus (Amersham Biosciences, Amersham, UK) per manufacturer directions. Briefly, cells were mixed with PBS 1:1 and loaded on top of Ficoll in 2:1. Cells were centrifuged and isolated in three layers and middle layer cells were removed carefully to new tubes and then washed twice with 10% FCS in PBS, re-suspended in culture medium. Trypan blue staining was used (Invitrogen Corp., Carlsbad, CA, USA) to assess for cell viability. Isolated BM consists of multiple populations including BM stem cells and mesenchymal cells.

Cells were then washed twice with 10% FCS in PBS, re-suspended in culture medium (see below). Trypan blue staining was used to assess cell viability. Whole BM progenitors were used in co-culture at 1×10^6 allogeneic BM cells/mL. The ratio of islet equivalent to BM cells was $1:10^4$. This was the optimum ratio given our preliminary studies.[11] Co-cultured cells were maintained in the same culture conditions as islet-only groups.

Human insulin assay
Insulin concentrations in the specimens (cell culture media or cultured tissue extracts) were measured using a Human Insulin ELISA Kit (Linco Research, St Charles, MO, USA) according to the manufacturer's instructions. Briefly, insulin standards and appropriately diluted (1:50–1:500) samples were added to an insulin Ab-coated 96-well microplate and incubated for 2 h at 4 °C. After five washings, anti-human insulin enzyme conjugate was added to each well and incubated for 30 min at room temperature. After seven more washings, enzyme substrate solution was added and then incubated for 45 min at room temperature in the dark. The reaction was halted by adding 1N sulfuric acid. Absorbance at 450 nm was measured with a µQuant microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA) and insulin concentrations calculated using KC Junior microplate reader software (Bio-Tek Instruments, Inc.).[18]

Immunohistochemistry
Cells on chamber slides were fixed with 3% paraformaldehyde, followed by exposure to 10% normal goat serum. Tissue was frozen in liquid nitrogen and frozen sections of 5 µM were cut. The slides were blotted without washing and primary Abs were applied. The slides were then incubated in a moist chamber at 4 °C overnight. The slides were washed three times, followed by exposure to the secondary Ab, for 45 min, at room temperature. After washing, diluted avidin-Texas Red (1:300; Molecular Probes, Eugene, OR, USA) was applied and the slides were incubated for 15 min. The slides were subsequently washed extensively with PBS and the above process repeated with a second fluorescent color and/or third Ag-detecting Ab. When the process was finished, slides were covered using a fluorescent mount medium and cover-slips. The samples were evaluated and photographed using confocal fluorescent microscopy.[19]

Angiogenesis protein array
Samples were analyzed by Human angiogenesis microarray (AB-PA3301, Capital Bioscience Inc., Gaithersburg, MD, USA). Human angiogenesis microarray kits provided spotted slides containing 16 identical arrays of 14 capture Abs in quadruplicate. Following the manufacturer's protocol, 50 µl samples were loaded onto each array and incubated while shaking for 2 h. Immediately afterward, loosely bound Ags were washed away and the array was incubated with biotinylated Ab for 45 min. Biotinylated detection Abs bind the Ags in a sandwich format. The biomarker detection is achieved with the addition of streptavidin-alkaline phosphatase conjugate, followed by alkaline phosphatase substrate for colorimetric development. The biomarker standards in the kit were used to quantify the abundance of the proteins in a sample. Positive and negative controls spotted within each array allow for assay validation.

Selected cytokine depletion
Cell culture quality Abs were used for depletion of BM released angiogenesis and growth factors: anti-human VEGF affinity purified polyclonal Ab, goat IgG (catalog no.: AF-293-NA), anti-human PDGF polyclonal Ab, rabbit IgG (catalog no.: AB-20-NA), anti-human KGF/FGF-7 affinity purified polyclonal Ab (catalog no.: AF-251-NA). Abs were purchased from R&D systems (Minneapolis, MN, USA).

Reverse transcriptase-PCR for islet cell gene expression

Total RNA was isolated from islet or islet and BM co-cultures using Trizol reagent (Invitrogen, catalog no.: 10296-028). Isolated RNA was treated with Turbo DNA-free (Ambion, Austin, TX, USA, catalog no.: AM1907) to remove genomic DNA. cDNA was synthesized from 500 ng of total RNA using Applied Biosystems TaqMan reverse transcription reagents kit (Applied Biosystems, Foster City, CA, USA, catalog no.: N808-0234) with random hexamers in a total volume of 100 μl according to the manufacturer's instructions. cDNA was stored at $-20\,^{\circ}C$ for downstream PCR. Quantitative real-time PCR was performed using Superarray SYBR green PCR kit (Superarray, Frederick, MD, USA, catalog no.: PA-012) in a total volume of 25 μl on Applied Biosystems 7000 sequence detection system (Applied Biosystems). The signal levels were normalized with GAPD for quantitative real-time PCR. Conditions for the PCR reaction were 10 min at $95\,^{\circ}C$, and then 40 cycles each consisting of 15 s at $95\,^{\circ}C$, 60 s at $60\,^{\circ}C$.

Image analysis and statistics

To evaluate islet size and vascularization, 20 images from each group were captured and images were analyzed with Image J software (http://rsb.info.nih.gov/ij/ Java based Image Processing and Analysis provided by NIH). The islet size correlation to islet vascularization was analyzed with two-way analysis of variance. Data were presented as mean ± 1 s.e. of the mean. In the text, tables and figures, all data are presented as means ± s.e.m. Data used for graphical presentation and statistical analysis are expressed as per experiment. However, the described experiments have been repeated three times. Data were analyzed by an analysis of variance statistics program using a two-factor analysis of variance of repeated measures. *Post hoc* comparisons among individual means were made by Tukey's *t*-test.

Results

BM initiated human islet vascularization in co-culture

Individual islets can be observed in a cluster-like structure 48 h after isolation (Figure 1A, image a and b). However, islets gradually lose their cluster appearance and reduce to a monolayer of cells after 2 months when cultured alone in normal culture conditions (Figure 1A, image c). When co-cultured with BM, human islets showed vessels surrounding the islet while islet-only cultures were monolayers (Figure 1A, image d). This indicates revascularization as a result of direct BM interaction.

After 2 months of culture, islet-only groups showed no signs of vessel growth (Figure 1B, image a). 4,6-diamidino-2-phenylindole staining (blue) indicates the presence of cells but immunohistochemistry staining for insulin (red) was sparse throughout the islet (Figure 1B, image a (top image)), suggesting a deficiency in insulin secretion. Islets co-cultured with BM showed apparent vessel growth (Figure 1B, image b). The presence of insulin (red) and CD31 staining for endothelial cells (green) shows vessel growth and insulin function in co-cultured islets

(Figure 1B, image b (top image)). These observations were apparent throughout all cultures.

BM paracrine function releases angiogenic and growth factors in co-culture

Protein array was used to evaluate levels of angiogenic and growth factors from human islet-BM co-culture and islet-only culture media (once a month from day 1 to 210) (Figure 2). Whole BM progenitors were used in co-culture. A total of 14 angiogenesis and growth factors were evaluated simultaneously. Among the angiogenic factors, VEGF-A levels from islets co-cultured with BM showed consistent elevations throughout the 210-day culture vs islet-only cultures, which showed almost null levels across several time points (Figure 2a). This was the same for growth factors PDGF and KGF in BM co-cultured islets vs islet-only cultures (Figures 2b and c). KGF levels were not detectable in either co-culture or islet-only culture media on day 1. However, a 30-fold increase was observed in co-culture vs islet-only culture media on day 30. Further increases in the levels of KGF were detected until day 60. This observation was consistent throughout the 210-day culture period except for a drop on day 152 (1.5-fold increase, Figure 2c). Despite low levels on day 1, TIMP-1 was consistently elevated from day 30 (twofold) until day 210. Islet-only cultures showed inconsistent fluctuations in TIMP-1 levels from day 60 to 152 (Figure 2d). The levels of angiogenin were consistently high in co-culture media vs the level in islet-only culture. The highest level was on day 60 (17-fold) (Figure 2e). Only angiopoietin-2 (angiopn-2) was shown to be lower in co-culture media as opposed to islet-only culture media except at the beginning of culture (co-culture to islet-only culture media 2:1). The levels in co-culture media remained lower than in islet-only culture media until day 150 (66-fold) (Figure 2f). The expression pattern of ICAM-1 in co-culture media appeared to be different from those of the other factors described above. High levels of ICAM-1 in the co-culture media were observed through day 60 vs the level in islet-only culture media. However, low expression of ICAM-1 in islet-only culture media were observed on day 152 (Figure 2f). All elevated levels of proteins in co-culture medium vs islet-only culture were significant ($P < 0.01$). Although not as marked as the factors mentioned above, the levels of EGF and FGF in the co-culture media were approximately 10% higher than in the islet-only culture media. The levels of HGF, IP-10, VEGFD, TIMP-2 and angiopn-1 exhibited no significant differences between the two culture media (data not shown here).

Selected depletion of BM angiogenic and growth factors hinders human islet vascularization

Islets co-cultured with BM showed increased vascularization (Figure 3A, top right). When angiogenesis factors KGF (Figures 3A, middle left and 3D), PDGF (Figures 3A, middle right and 3D), KGF + PDGF (Figures 3A, bottom left and 3D), and VEGF (Figures 3A bottom right and 3D) were depleted using Abs, the occurrence of vascularization decreased significantly (Figures 3B and D). Depletion of VEGF alone was shown to have the most significant effect in
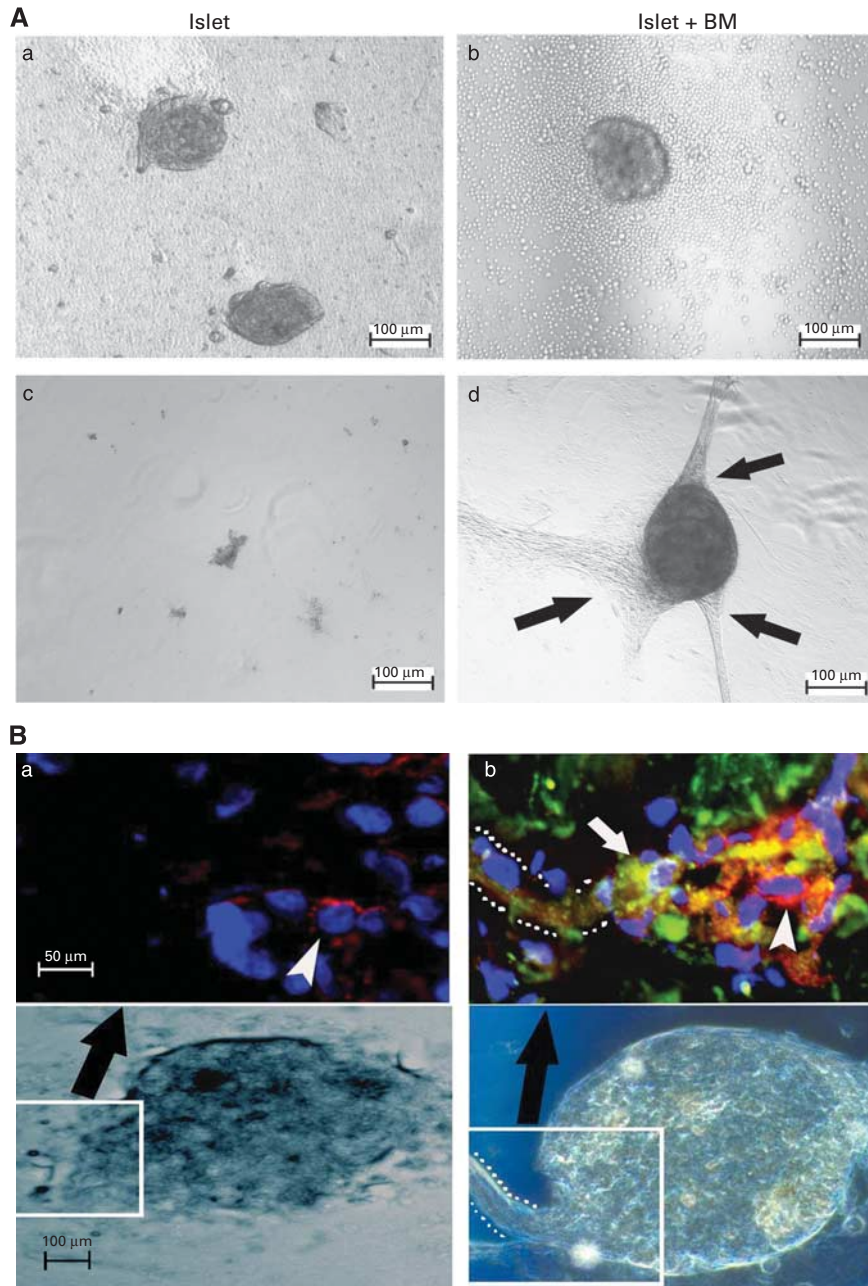
Figure 1 (**A**) Human islets co-cultured with BM for 2 months under light microscopy: (a) Human islet culture at day 2 and (b) culture with BM at day 2 display normal islet morphology. (c) After 2 months, islet-only cultures displayed dispersed monolayer cells. (d) Human islets with BM not only generated vessels but also increase islet size (indicated by arrows). Magnification × 10. (**B**) BM promotes human islet vascularization after 2 months. No vessel-like tissue was observed in islet-only culture (panel a bottom, light microscopy image) and selected square area in fluorescence immunohistochemistry (respective top image indicated by black arrow) shows only weaker insulin staining (red with anti-insulin Ab, indicated by small white arrow head), nuclei staining with 4,6-diamidino-2-phenylindole (DAPI) (blue). Islet with BM co-culture shows significant vessel-like tissue generated (boxed in panel b, bottom) and stronger insulin staining (white arrow head indicated in respective top image) and endothelium staining (green with anti-CD31 Ab, indicated by white arrow). Detection of insulin (red) within the dotted lines (panel b top image) suggests insulin release into vessels from β-cells in islets. Magnification × 20 (bottom images); magnification × 60 (top images).

reducing vessel growth (Figures 3B and D). Morphologically, BM islets depleted with VEGF Ab showed very little insulin function (red) and CD31 (green) (Figure 3C, top left) vs BM islets without Ab depletion, as indicated by the strong presence of insulin (red) and CD31 (green) (Figure 3C, top right). In addition, islets co-cultured with BM had an overall significant size increase vs islets without co-culture (Figure 3D).
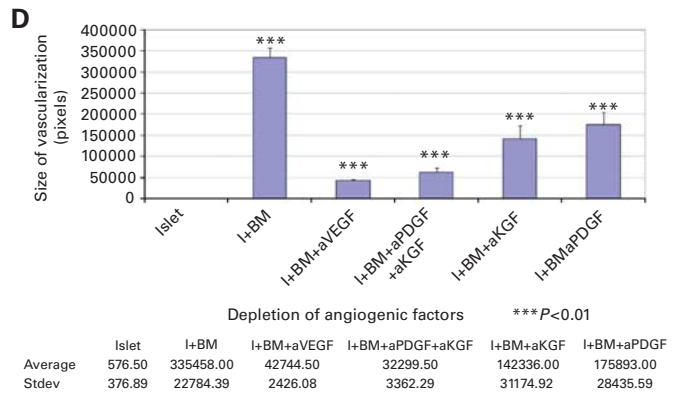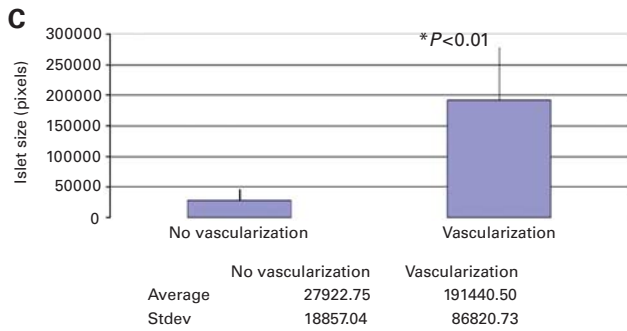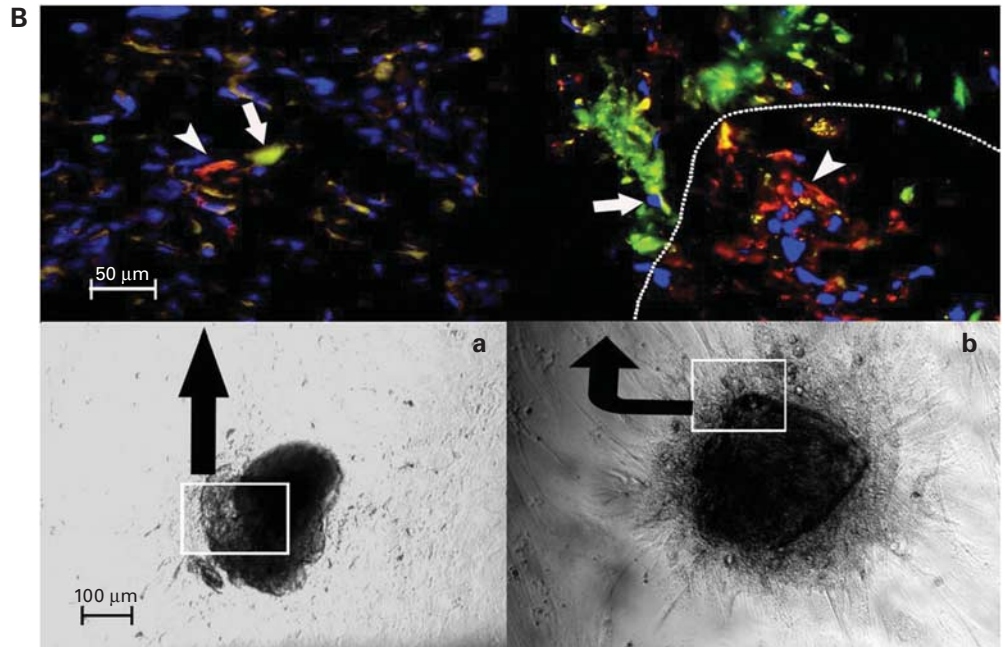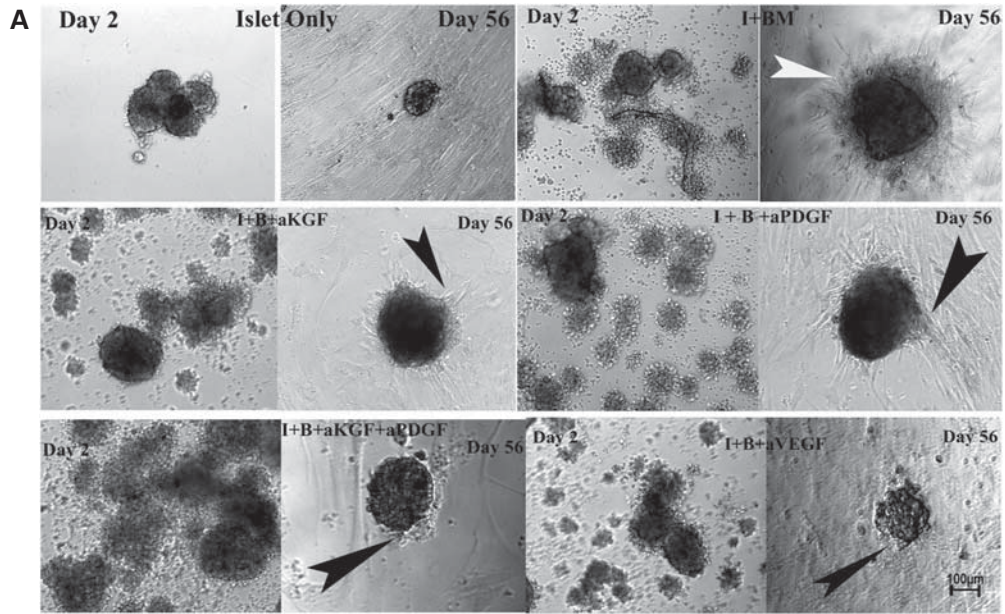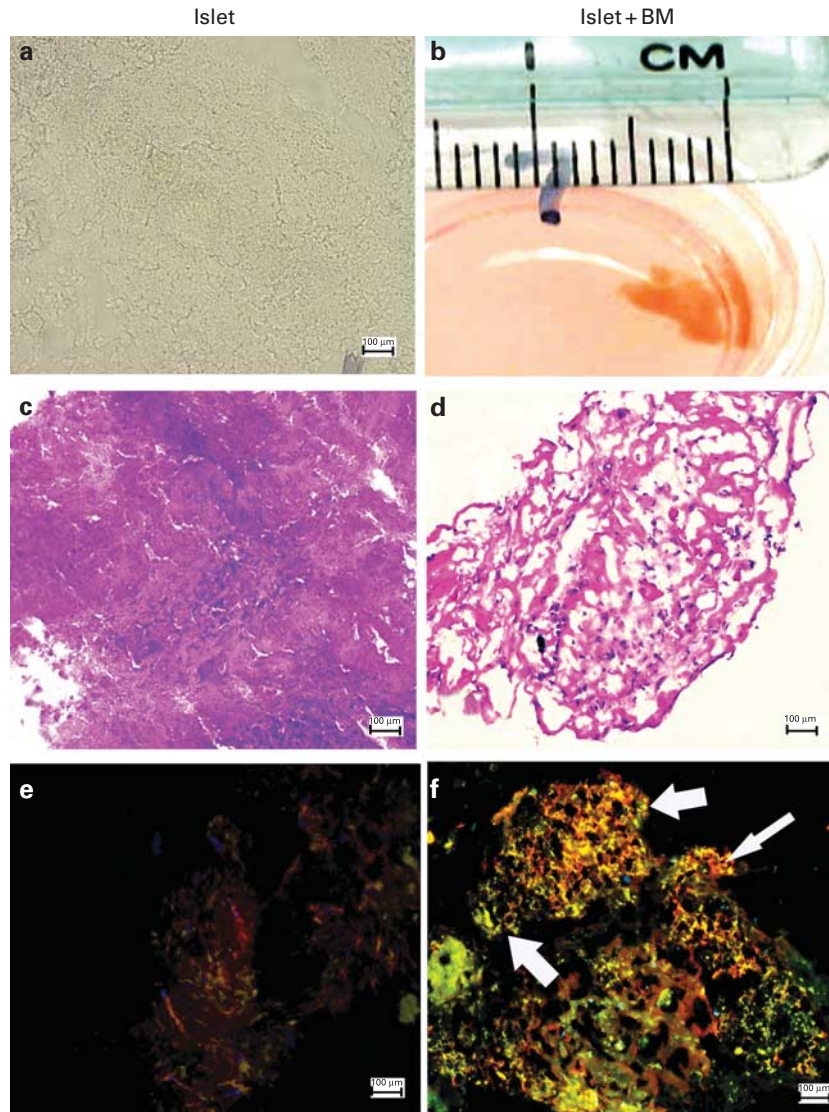
BM reconstitutes human islet tissue
After 6 months of culture, islet-only groups showed monolayer morphology (Figure 4a) vs BM co-cultured
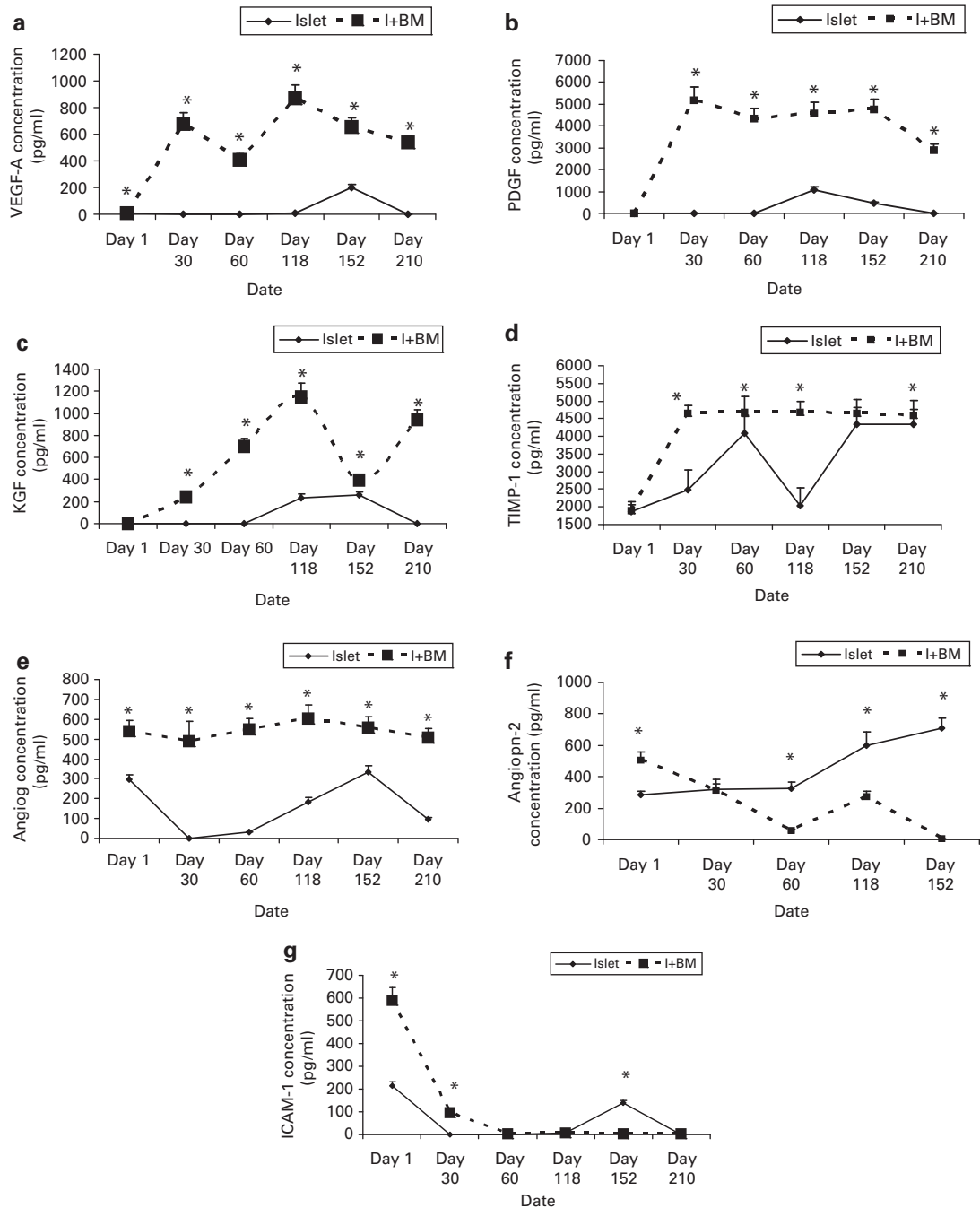
Figure 2 A total of 14 growth factors related to angiogenesis in culture medium were evaluated by protein array. Seven factor levels were found to have significant differences in the medium of BM culture with human islet vs islet-only culture, *$P<0.01$ and $n=6$. The panels show angiogenic and growth factor levels of BM co-culture with islet (I + BM) and islet-only for 210 days. (**a**) VEGF-A, (**b**) PDGF, (**c**) KGF, (**d**) TIMP-1, (**e**) angiog, (**f**) angiopn-2 and (**g**) ICAM-1.

Figure 3 (**A**) Top panel shows human islet culture and islets co-cultured with BM cells on day 2 and 56. Arrow indicates vascularization in BM co-cultured islets (top images); middle panel shows islets with BM culture with Abs for KGF and PDGF: islet vascularization was less apparent in islet-only vs islets cultured with BM; bottom panel shows co-culture with Abs for KGF/PDGF and VEGF, which resulted in significantly less islet vascularization (a = Ab). (**B**) (a) Human BM culture with VEGF depletion on culture day 56 β-cells and endothelioid cells in islets (top) were identified by anti-insulin Ab (red, arrow head indicated) and endothelioid cells were identified by anti-CD31 Ab (green, arrow indicated). Cellular nuclei were stained by 4,6-diamidino-2-phenylindole (DAPI) (blue). Top image shows a three-dimensional architecture (indicated by arrow). (b) Islets from BM culture without cytokine depletion are circled by white dotted lines. Endothelioid cells are indicated by arrows and β-cells are indicated by arrow head. Magnification × 20 (bottom images); magnification × 60 (top images). (**C**) Using two-way analysis of variance (ANOVA) analysis on the human islet size correlation to islet with and without vascularization. $n=20$, *$P<0.01$. (**D**) Effects of BM released cytokines on human islet growth after 48-day culture, human islet size was measured by image unit (pixel) and cytokine depletion groups compared with I + BM using two-way ANOVA. $n=20$, ***$P<0.001$. a = Ab.

A

B

C

	No vascularization	Vascularization
Average	27922.75	191440.50
Stdev	18857.04	86820.73

D

Depletion of angiogenic factors   ***P<0.01

	Islet	I+BM	I+BM+aVEGF	I+BM+aPDGF+aKGF	I+BM+aKGF	I+BM+aPDGF
Average	576.50	335458.00	42744.50	32299.50	142336.00	175893.00
Stdev	376.89	22784.39	2426.08	3362.29	31174.92	28435.59

Islet   Islet + BM

Figure 4 The images show islet-only (**a, c, e**) and islet with BM (**b, d, f**) after 6 months culture. (**a**) Islet-only culture became monolayer; (**b**) BM co-cultured islets maintained tissue morphology and grew to a tissue size around 0.25 cm^2; (**c**) Stained with hematoxylin and eosin (HE), islet-only culture show high-density cell staining but a lack of islet-like structure, (**d**) BM cultured islets show islet-like structure surrounded by porous tissue; (**e**) Fluorescence immunohistochemistry evaluated β-cell (red) and endothelia (green). Islet-only culture tissue appeared to have no significant positive staining; (**f**) BM co-cultured islets with β-cell (red with anti-insulin Ab, indicated by small arrow) and endothelioid cells (green with anti-endothelial cell Ab, big arrow indicated); (**a, c, d, e, f** magnification ×10, (**b**) indicated by ruler).

groups, which generated human islet tissues ranging from 0.1 to 1.5 cm^3 in a three-dimensional shape (Figure 4b). Morphological analysis of the islet tissue using hematoxylin and eosin stain showed a porous construction with many inter-tissue spaces throughout the entire islet (Figure 4d). On the other hand, the islet-only culture tissue contained a solid monolayer mass without inter-tissue space (Figure 4c). Further studies with fluorescence immunohistochemistry revealed significant populations of endothelial cells inside the islet tissue with positive insulin staining cells as indicated by CD31 staining (green). This was also coupled with the presence of insulin function (red) (Figure 4f). Insulin function in terms of secretion into culture medium was consistent with our previous reports. BM co-cultures exhibited significantly higher levels of insulin secretion vs islet-only cultures throughout culture.[11]

Allogeneic BM supports human islet function by sustaining islets in culture
Islets co-cultured with BM showed strong expression of insulin (red) and glucagon (green) using immunohisto-chemistry, which indicates islet function (Figures 5A and B) in comparison with islet-only groups that did not express insulin or glucagon (Figure 5Aa). Gene expression levels were significantly elevated for glucagon (GCG 24.4-fold vs control) and insulin (INS 28.66-fold vs control) (Figure 5Ac). Gene expressions for PDX-1 (5.46-fold vs control), a pancreatic β-cell developmental gene and NK × 2.2
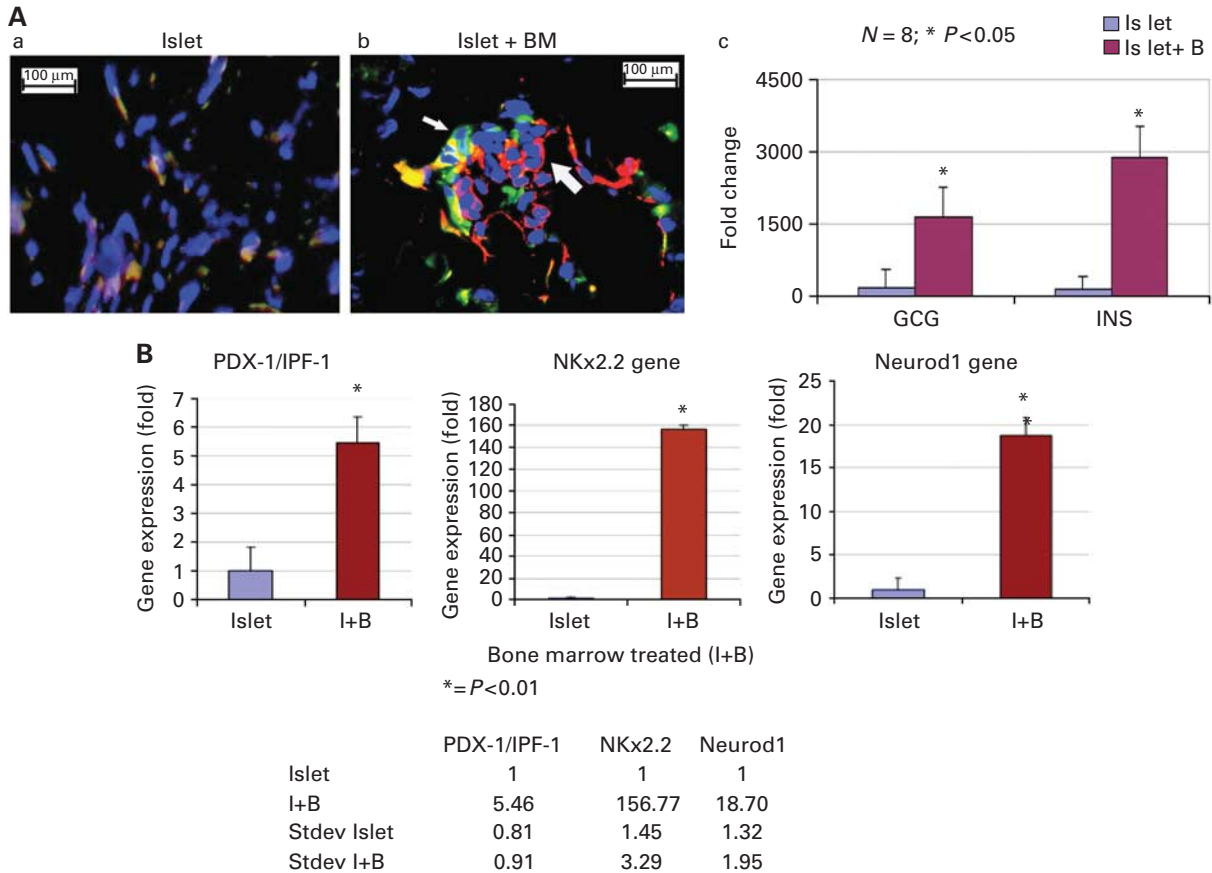
Figure 5 (A) Fluorescence immunohistochemistry to identify α- (glucagon, green) and β- (insulin, red) cells and their gene expressions in long-term culture (6 months). (a) Islet-only culture: although there are numerous cells identified by nuclear staining (blue, 4,6-diamidino-2-phenylindole (DAPI)), there was no positive staining for α- and β-cells; (b) BM cultured islet tissue sustained not only islet structure but also β-cells (red, large arrow) and α-cells (green, small arrow); (magnification ×40); (c) significantly high levels of α- and β-cell gene expressions in BM culture islet tissue; INS = insulin gene; GCG = glucagon gene; *P < 0.01, n = 8. (B) Reverse transcriptase (RT)-PCR analysis for β-cell regeneration transcription factor gene expressions show that PDX, Nk × 2.2 and NeuroD 1 gene expressions were significantly increased in co-culture vs islet-only culture.

(156.77-fold vs control)/NeuroD1 (18.7-fold vs control), β-cell transcription factors, were upregulated by BM co-culture. This indicates potential β-cell proliferation under the influence of BM.

Discussion

In vitro, allogeneic BM supports human islet survival and function in long-term co-cultures.[11] However, the mechanism remains unclear. It is also interesting that BM progenitor subtypes (endothelial and mesenchymal cells) do not benefit islet function as much as whole BM progenitors (data not shown) in the long term. In previous studies, we were not able to observe the same benefits from BM-conditioned medium or a Netwell-isolated BM culture system. This indicates that BM support human islets in a unique way. In this study, we observed that BM paracrine activity initiates human islet angiogenesis, repairs islet injury incurred by the isolation process and stimulates human islet regeneration. By utilizing a protein array technique to evaluate BM and islet co-culture media, we

found that BM released multiple angiogenesis and growth factors such as VEGF-A, PDGF and KGF in long-term co-cultures (Figure 2). High levels of these angiogenic and growth factors in BM and human islet co-cultures are believed to support and stimulate human islet vascularization and tissue regeneration. Maintaining high levels of these three factors simultaneously may represent synergistic activation of human islet vascularization and tissue regeneration. We showed that it is a combination of factors that promote human islet vascularization and growth. Indeed, the selected depletion of individual Abs for VEGF, PDGF and KGF significantly affected islet reconstitution but did not completely abolish BM's effect on human islets. Future studies are needed to identify whether administering these factors alone may cause islet angiogenesis and regeneration. However, these factors may only be a component of BM's effects. Although we did not test of the effects of ICAM-1 and angiopn-2 on initiating tissue repair, ICAM-1 and angiopn-2 have been known to initiate cell–cell contact, repair cell injury in response to apoptosis and be involved in vascular inflammation and islet carcinoma.[20–23] Significantly high levels of ICAM-1 at the

beginning of culture and low levels in late culture suggest that this factor may participate in sustaining human islet viability. Future studies are needed to confirm this hypothesis. High levels of angiopn-2 in islet-only culture vs BM co-culture suggest that angiopn-2 can be released from human islets, which has been known to result in human islet carcinoma[24] and inflammation.[23] However, human islets co-cultured with BM had no detectable levels of angiopn-2, suggesting that BM inhibited this particular factor in co-culture. These data suggest that BM co-cultured human islets have the potential to selectively prevent tumor generation and islet inflammation.

Human islet angiogenesis includes both tissue generating vessels and microvascular vessels inside the islet tissue, supporting islet cell endocrine function. Through re-vascularization, human islet cells can increase the absorption rate of nutrition and disposal of waste *in vitro*. Increased vascularization was prominent in BM cultures as marked by an increase in vessel proliferation (Figure 1) and consequently an increase in the overall size of the BM co-cultured islet tissue (Figure 3). This is important for islet tissue survival after transplantation. BM's ability to initiate human islet microvascularization and tissue regeneration appears to be a novel mechanism in BM supporting human islet function. BM has the ability to stimulate angiogenesis and islet survival via several growth related factors as well as to restrict potential tumor inducing factors in co-cultures further supports the promising effects of BM on human islets. However, further studies are needed to identify how cell–cell contact contributes to BM paracrine effects in initiating islet cellular signal pathways for islet re-vascularization and growth.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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