Switching-on Survival and Repair Response Programs in Islet Transplants by Bone Marrow-derived Vasculogenic Cells.

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**Objective:** Vascular progenitors of bone marrow (BM) origin participate to neo-vascularization at sites of wound healing and transplantation. We hypothesized that the biological purpose of this BM-derived vascular component is to contribute angiogenic and survival functions distinct from those provided by the local tissue-derived vasculature.

**Research Design and Results:** To address this hypothesis, we investigated the functional impact of BM-derived vascular cells on pancreatic islets engraftment using BM-reconstituted Id+/+-Id3-/- mice, a model of BM-derived vasculogenesis. We show that, in this model, BM-derived vasculogenic cells primarily contribute to the formation of new blood vessels within islet transplants. In contrast, grafts re-vascularization in a wild type background occurs by tissue-derived blood vessels only. Using these distinct transplant models in which BM- and tissue-derived vasculature are virtually mutually exclusive, we demonstrate that BM-derived vasculogenic cells exhibit enhanced angiogenic functions and support prompt activation of islets survival pathways which significantly impact on islets engraftment and function. Moreover, gene profiling of vascular and inflammatory cells of the grafts demonstrate that neo-vascularization by BM-derived cells is accompanied by the activation of a genetic program uniquely tuned to down-regulate harmful inflammatory responses and promote tissue repair.

**Conclusions:** These studies uncover the biological significance of BM-derived vasculogenic cells in the response to injury during transplantation. Enhancing the contribution of BM-derived vasculogenic cells to transplantation sites may help to overcome both limited angiogenic responses of the adult tissue-derived vasculature and untoward effects of inflammation on transplant engraftment.
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Tissue repair after wounding and/or cell transplantation requires the concerted regulation of angiogenic and self-limited local inflammatory responses (1). Several studies have shown that angiogenic responses involve both tissue-derived vascular cells as well as circulating BM-derived vascular precursors (2-9). The potential of these progenitors to develop into endothelial and/or perivascular cells supporting tumorigenesis and tumor growth has been extensively documented (2-6,9-15). In the context of primary tissues, a much more limited incorporation of these cells into injured blood vessels has been observed, suggesting a role in vascular repair (2,7,8). Whether in this instance the angiogenic response of such BM-derived vascular component is distinct from that supported by pre-existing tissue-derived blood vessels, and/or contributes a biological advantage, is presently unknown. Furthermore, the potential of BM-derived vascular cells to influence local inflammatory responses normally associated with angiogenesis, has not been addressed.

Pancreatic islet transplants are highly sensitive to the efficiency of revascularization, since defects of this process result in rapid cell loss and/or altered response of the grafts to glucose (16-19). Thus, islet transplants provide an ideal model to assess the impact of BM-derived vascular cells on both survival and function of a primary tissue. Hence, we hypothesized that BM-derived vasculogenic cells, recruited at sites of pancreatic islet transplantation, contribute engrafting and survival functions distinct from those provided by the endothelium sprouting from the pre-existing vasculature. To address this hypothesis we used the BM-reconstituted Id1+/Id3-/- mouse, a model of BM-derived vasculogenesis (9,20). Id1 and Id3 transcription factors regulate vascular and neuronal cell differentiation and proliferation (21). Accordingly, Id1-/-Id3-/- knock-out mice exhibit severe vascular malformations, including absence of sprouting and branching of blood vessels (20). In contrast, Id1+-Id3-/- mice, maintaining one functional Id1 allele, display no overt vascular defects. However, pre-existing blood vessels in these mice fail to mount an efficient angiogenic response to support tumor transplant engraftment (9). Interestingly, reconstitution of Id1+/Id3-/- mice’s hemopoietic compartment with wild type BM rescues tumor revascularization (9). Moreover, in these BM chimeras, more then 90% of the endothelium forming new blood vessels in the tumor implants is derived from wild type BM, indicating that neo-vascularization is sustained almost entirely by BM-derived endothelial precursors.

Here, we exploited this model for islet transplantation and demonstrate that the majority of new blood vessels formed at the site of islet transplantation in BM-reconstituted Id1/Id3 deficient mice are of BM origin. In contrast, revascularization of islet transplants in BM-reconstituted wild type (WT) recipients occurs predominantly by tissue-derived blood vessels. Using these transplantation models, we provide evidence that tissue- and BM-derived vasculatures are not functionally equivalent in that development of BM-derived blood vessels is associated with enhanced angiogenesis and with the activation of survival and cellular pathways uniquely skewed toward protective inflammatory responses and tissue repair.

RESEARCH DESIGN AND METHODS

Animals and BM reconstitution. Id1+/Id3+-/- and WT mice were bred at TSRI pathogen-free facility and Id1+/Id3+-/- mice screened as described (20). C56BL/6-TgN(ACTBEGFP)1Osb, ROSA26 (Jackson...
Laboratory) or WT mice were used as BM donors. BM cells were flushed from femurs with RPMI-10% FCS, depleted of CD3+ T cell using magnetic beads (Miltenyi Biotech) and injected I.V (5-10x10^6) in 6-8 weeks old lethally irradiated WT and Id1+/-Id3-/-mice (1200 rads). After 6 weeks, BM reconstitution was assessed by flow cytomerty of peripheral blood to identify GFP+ cells, or β-gal+ cells stained with fluorescein-β-D-galactopyranosyde (FDG, Molecular Probes).

**Islet isolation and transplantation.** Islets were isolated by intra-ductal injection of Liberase (0.5 mg/ml) and purified on a Ficoll gradient. Islets were cultured overnight in RPMI-10% FCS, hand picked and transplanted under the kidney capsule. Diabetic mice were generated by IP injection of a single dose of streptozotocyn (200mg/Kg) (22), and 1 week later transplanted with islets. Upon ensuing of frank hyperglycemia (i.e. 200 mg/dl), mice were injected subcutaneously with insulin (Humulin L, Lilly) up to 3 days post-transplant. In normoglycemic mice the graft’s function was verified by the return to hyperglycemia upon removal of the graft.

**Histology.** To identify functional blood vessels, mice were injected IV with FITC-ISB4 (200μg, Molecular Probes) prior to euthanasia. Tissues were immuno-stained as described (8) using the antibodies listed in the Online Appendix. Apoptotic cells were detected by TUNEL using a digoxigenin-labeling kit (Chemicon). Sections were visualized at a Zeiss Axiovert microscope equipped with a scanning laser confocal attachment (Radiance-2000; Biorad) or at a NIKON Eclipse-800 Microscope, equipped with a Spot II CCD camera. Morphometric analysis were performed on ~20-30 sections/graft collected at 100 μm intervals till exhaustion of the grafts, using the Spot Advanced and ImageProPlus softwares.

**Cell separation and flow cytometry.** After *in vivo* injection of FITC-ISB4, grafts were micro-dissected and dissociated at 37 C in HBSS/Liberase (0.5mg/ml)/DNase I (50μg/ml) followed by non-enzymatic dissociation medium (Sigma). Cells were then incubated with primary and secondary antibodies as detailed in the Online Appendix. For cell isolation, single cells dissociated from biotin-ISB4-perfused grafts (n=5) were labeled with primary Abs followed by RPE-conjugated secondary reagents and anti-PE microbeads (Miltenyi Biotech) as detailed in the Online Appendix. Cells were positively selected on magnetic columns and analyzed at a FACSscan (Becton Dickinson). CD31+ and F480+ cells were >90% pure, whereas purity of ISB4+ fractions was >75%.

**Western Blotting and pAkt [S473] ELISA.** Cells were lysed in 10 mM Tris, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na_2PO_4, 2 mM Na_3VO_4, 1% TX-100, 0.1% SDS, 0.5% deoxycholate, 1 mM PMSF and Complete (Roche). Equal amounts of proteins were loaded on 4-12% SDS-PAGE gels, transferred to PVDF membranes and probed with mouse anti-PML (clone MAB3738, Chemicon), goat anti-adipsin (Santa Cruz), or anti-S100A8 (R&D) antibodies. Antibody binding was revealed using an ECL-based detection system (Kirkegaard and Perry Labs). Phosphorylated pAkt[S473] and total Akt were measured by ELISA (Biosource) using five micrograms of proteins.

**RNA extraction and DNA microarrays.** RNA was extracted using RNeasy kit (Quiagen). Biotinylated cRNA was prepared using the Illumina RNA Amplification kit (Ambion, Inc.) and microarray experiments performed as detailed in the Online Appendix.

**Statistical analysis:** Statistical analysis of the data between naïve and BM-reconstituted Id1+/-Id3-/- mice, or between BM-reconstituted Id1+/-Id3-/- and WT mice, was performed using the Student t-test and data comparing more than two groups was...
valuated by Anova followed by Bonferroni posttest. Data with P values <0.05 were considered statistically significant.

RESULTS
Failure of pancreatic islet engraftment in the Id1/Id3 deficient mice and rescue by BM reconstitution. To evaluate whether BM reconstitution of Id1+/−/Id3−/− mice supports engraftment of pancreatic islets as seen for tumor grafts (9), Id1+/−/Id3−/− and WT mice were reconstituted with WT BM. In order to track BM-derived cells, GFP-transgene or ROSA26 mice were used as BM donors. Untreated Id1+/−/Id3−/− mice were used as controls. FACS analysis at 6 weeks post-BM transplantation demonstrated that >80% of the leukocytes in BM-reconstituted Id1+/−/Id3−/− and WT mice were GFP or β-gal+, i.e. of BM-donor origin. Furthermore, Colony-Forming Unit (CFU) assays and FACS analysis demonstrated that all hemopoietic lineages were reconstituted to a normal range in both WT and Id1+/−/Id3−/− hosts (not shown).

Six weeks after BM engraftment, the mice were transplanted under the kidney capsule with 500 WT pancreatic islets. Immunostaining for insulin at 1 week post-transplantation demonstrated that untreated Id1+/−/Id3−/− mice harbored significantly smaller grafts than BM-reconstituted WT mice (Figure 1A). However, reconstitution with WT BM rescued engraftment in Id1+/−/Id3−/− mice (Figure 1B). Furthermore, the insulin+ area of the grafts from BM-reconstituted Id1+/−/Id3−/− mice was >2.5 fold larger than that of grafts from BM-reconstituted WT mice (Figure 1B, P<), suggesting differential islet cell survival. Morphometric analysis demonstrated a ~2 fold increase in the number of TUNEL+ apoptotic cells in islet grafts of untreated Id1+/−/Id3−/− mice as compared to BM-reconstituted WT or Id1+/−/Id3−/− mice (Figure 1C). In contrast, the number of apoptotic cells in the grafts of BM-reconstituted WT and Id1+/−/Id3−/− mice was not significantly different. Even at earlier time points (i.e. 2 days), TUNEL+ islet cells were very rare in these two groups of mice (i.e. <0.1%), possibly due to efficient clearance of apoptotic cells.

Signaling by Akt positively regulates cell survival and proliferation (23, 24). Therefore, we investigated whether this pathway was differentially activated in grafts of BM-reconstituted WT versus Id1+/−/Id3−/− mice. Measurement of Akt activation by detection of pAkt[S473] in islet cell lysates revealed significantly higher levels of pAkt[S473] in BM-reconstituted Id1+/−/Id3−/− versus WT mice (Figure 1D). Furthermore, grafts immunostaining demonstrated a strikingly different pattern of pAkt[S473] expression in situ. Thus, while in BM-reconstituted Id1+/−/Id3−/− mice a strong immunoreactivity for pAkt was observed throughout the graft, in WT controls only cells at the periphery of islets were strongly positive for pAkt (Figure 1E-F, arrows). In both grafts, pAkt[S473] highlighted primarily cell nuclei and, to a lesser extent, the cytoplasm, two known localizations of activated Akt (25). The preferential nuclear localization of pAkt in the transplants of BM-reconstituted Id1+/−/Id3−/− resembled that of tumors lacking the promyelocytic leukemia antigen (PML), a tumor suppressor gene regulating cell proliferation and apoptosis (26). Consistent with the function of PML as negative regulator of Akt activation and nuclear localization (26), immunostaining for PML in islet grafts demonstrated a pattern of expression which mirrored pAkt[S473], i.e., weak labeling of islet cells in BM-reconstituted Id1+/−/Id3−/− mice, and strong labeling of islets in WT recipients (Figure 1G). Immunoblotting of islets protein extracts confirmed down-regulation of PML in BM-reconstituted Id1+/−/Id3−/− versus WT mice (Figure 1H). In both grafts, no cycling endocrine cells were detected (not shown), indicating that pAkt expression and its
preferential nuclear localization were linked to cell survival rather than proliferation.

Thus, rescue of transplant engraftment by BM reconstitution in Id1+/-Id3-/- mice is associated with decreased islet cell death and activation of survival signals.

**Characterization of the grafts' vasculature.**

Whole grafts of BM-reconstituted Id1+/-Id3-/- mice at 1 week post-transplant displayed ~ a two-fold higher density of PECAM-1+ blood vessels than those of either untreated Id1+/−Id3-/- or BM-reconstituted WT mice (Figure 2A-B). PECAM-1+ blood vessels in the grafts of BM-reconstituted Id1+/-Id3-/- mice also appeared more branched than those of WT controls (Figure 2A, insets). As compared to the endocrine component, the non-endocrine (i.e. connective and inflammatory) tissue was more abundant in WT recipients (Figure 1A) and less vascularized. Morphometric analysis of blood vessels within the grafts endocrine component only, showed a ~25% increase of vascular density in BM-reconstituted Id1+/-Id3-/- versus WT mice (n=3) versus WT mice (n=4) (Figure 2C).

The lower vascular density in the grafts of WT recipients could not be attributed to irradiation, since the density of PECAM-1+ blood vessels was not significantly different in irradiated and non-irradiated recipients (i.e. = 13.3 ± 0.25 % versus 14.1 ± 1.3 %, mean ± SEM, n=4). Furthermore, staining with the Meca-32 antibody, preferentially labeling arterioles and venules over capillary endothelia (27), demonstrated that the density of Meca-32+ blood vessels was only slightly increased in BM-reconstituted Id1+/-Id3-/- versus WT mice (i.e. 12.9 ± 0.2% versus 11.1 ± 0.4 %, mean ± SEM, n=3). Thus, at 1 week post-transplantation, the increased vascular density of islet grafts in BM-reconstituted Id1+/-Id3-/- appears to be mainly accounted for by the development of a PECAM-1+ capillary network.

At 4 weeks post-transplantation, a time when revascularization of islet grafts is completed (16,17), whole grafts’ vascular density was similar in BM-reconstituted Id1+/-Id3-/- and WT recipients (Figure 2B); however, in the endocrine component of the grafts, it was still ~ 25% higher in BM-reconstituted Id1+/-Id3-/- (n=3) as compared to WT mice (n=4) (Figure 2C). When compared to islets endogenous to the pancreas, the grafts’ vascular density in BM-reconstituted Id1+/-Id3-/- mice was remarkably similar at 1 week post-transplantation, whereas in WT mice it was always less than that of endogenous islets (Figure 2C).

BM-reconstituted Id1+/-Id3-/- mice also efficiently re-vascularized islets isolated from Id1+/-Id3-/- donors (Figure 2C). We hypothesized that, due to the growth defects of Id1/Id3 deficient endothelial cells (9,20,21,28), these transplants would unlikely be supported by islet donor-derived endothelial cells which might survive islet isolation (29,30). Under these conditions, 2 out of 3 grafts failed to engraft in WT recipients. In contrast, all transplants in BM-reconstituted Id1+/-Id3-/- mice engrafted and, at 4 weeks post-transplant, displayed ~2-fold higher vascular density than the single transplant engrafted in WT mice (Figure 2C).

In vivo injection of FITC-labeled Isolectin-B4 (ISB4) demonstrated that blood vessels developing in BM-reconstituted WT and Id1+/-Id3-/- mice, but not those in untreated Id1+/-Id3-/- mice, were patent (Figure 2D). ISB4+ blood vessels co-expressed the endothelial marker PECAM-1 but not the pan-leukocyte antigen CD45 (not shown). FACS analysis of cells dissociated from the grafts showed that in WT mice 78% and 89% of PECAM-1+CD45+ cells displayed bound ISB4 at 1 and 4 weeks post-transplantation, respectively (Online Appendix Figure S1C), whereas in BM-reconstituted Id1+/-Id3-/- mice a higher proportion of PECAM-1+CD45+ endothelial cells were ISB4+ at either time points.
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(i.e. 91% and 98% respectively, Figure S1D). These results indicate that, while the majority of PECAM-1+ cells detected in situ outline functional vessels, some do not. This latter fraction, possibly part of developing vascular structures yet not blood-perfused, is higher at 1 than at 4 weeks post-transplantation in either grafts and in the grafts of WT recipients at either time points.

Immunostaining for β-gal in islet grafts from Id1+/-Id3-/- mice reconstituted with ROSA26 BM demonstrated that the majority of ISB4+ blood vessels were β-gal+, indicating their BM origin (Figure 2E). In contrast, BM-derived β-gal− endothelial cells were virtually undetectable in WT recipients reconstituted with ROSA26 BM. Ultrastructurally, BM-derived blood vessels appear as small capillaries lined by a fenestrated endothelium and perivascular cells (Online Appendix, Figure S2A), reminiscent of islets’ capillaries in the endogenous pancreas (31). In contrast, blood vessels in the grafts of BM-reconstituted WT mice appeared larger and lined by a fenestrated endothelium loosely attached to the basal membrane (Figure S2B, asterisks).

These results demonstrate that enhanced survival and engraftment of islet transplants in BM-reconstituted Id1+/Id3-/- mice correlates with a predominant BM-derived vasculogenic component and rapid development of a functional, dense capillary network. In contrast, new blood vessels in WT mice are formed primarily from the pre-existing tissue-derived vasculature and expand less efficiently into blood-perfused capillaries.

**Grafts re-vascularization by BM-derived endothelium is associated with enhanced recruitment of GR1highF480+ inflammatory cells, and with the activation of repair response genes.** At 1 week post-transplantation, many CD45β-gal+ inflammatory leukocytes were observed in the grafts of mice reconstituted with β-gal−BM (Online Appendix Figure S3). Peri-islets inflammatory cells were present in both Id1/Id3-deficient and WT recipients and comprised myeloid F480+ cells (Figure 3A). FACS analysis demonstrated a ~2.5 fold increase in F480+ and GR1highF480+ cells in the grafts, but not BM compartment, of BM-reconstituted Id1+/-Id3-/- mice versus WT controls (Figures 3B-3C, n=4, *p*<0.002). Numbers of T and B cells were similar in the two grafts at 1 week post-transplantation, whereas grafts from Id1/Id3-deficient mice had fewer T cells at 4 weeks post-transplantation (Figure 3D, n=2).

To gain insights into the genetic program activated within the distinct vascular and inflammatory transplant microenvironments, in Id1/Id3-deficient and WT mice, we performed gene screening experiments using RNA from whole grafts as well as from myeloid and endothelial (i.e., CD31+ and ISB4+) cells isolated from the grafts at 1 week post-transplantation. Analysis of transcripts exhibiting >2-fold changes in BM-reconstituted Id1+/-Id3-/- versus WT mice demonstrated differential expression of inflammation and angiogenesis-related genes (Table 1 and Figure 4). First, a significant decreased expression of complement components and adipokines, including adipin, adiponectin and leptin, was observed in whole grafts from BM-reconstituted Id1+/-Id3-/- mice. Conversely, genes regulating the influx and function of neutrophils, monocytes and eosinophils (e.g CCL1, CCL2, CCL7, Calgranulin A and B) were up-regulated in CD31+ and ISB4+ samples of BM-reconstituted Id1+/-Id3-/- mice. Most remarkably, in these samples we observed an increased expression of genes marking macrophages polarized toward Type II immune responses (e.g. RELMα, IL10, Chitinase 3-like molecules, CCL24, SLAM, Arginase I) (32), as well as anti-inflammatory genes involved in the response to pathogens, oxidative stress and wound...
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healing (e.g., trefoil factors, serine peptidase inhibitors, cathelicidin, glutathione peroxidase 2) (33,34). Angiogenesis-related genes were also up-regulated in Id1/Id3 deficient versus WT mice and included: adhesion receptors to extracellular matrix (ECM) proteins (Thy-1, Spondin 2, SPARC), pro-angiogenic ECMs and remodeling enzymes (pro-collagen Type VI, MMP7, TIMP1) and molecules regulating endothelial cell proliferation (CXCR3, IL6) (Figure 4). Differential protein expression of select genes was confirmed by immunohistochemistry, immunoblotting and/or ELISA (Online Appendix Figure S4).

These findings demonstrate that, compared to the tissue-derived vasculature, BM-derived vascular cells are associated with an inflammatory component enriched for myeloid cells and functionally skewed toward protective immune responses and tissue repair.

Islet transplants re-vascularized by BM-derived vasculogenic cells readily reverse diabetes in vivo. To evaluate the translational implications of the enhanced angiogenic and repairing functions associated with BM-derived vasculogenic cells at sites of islet transplantation, we induced diabetes in the Id1/Id3 deficient strain by streptozotocin and then assessed the ability of islet transplants to reverse hyperglycemia. Streptozotocin-injected Id1+/-Id3-/- and WT mice became hyperglycemic by 72 hours post-injection and remained diabetic up to a 4 week follow up period (Figure 5A). After this time point the experiment was ended as diabetic mice would not survive without treatment. Next, WT and Id1+/-Id3-/- mice were reconstituted with WT BM, rendered diabetic by streptozotocin, and 1 week later transplanted with either 500, 200 or 75 islets. Daily monitoring of blood glucose demonstrated that transplants of 500 islets effectively restored normoglycemia in all mice within 1 week post-transplantation (Figure 5B). Interestingly, transplants of 200 and as few as 75 islets were also sufficient to rapidly restore normoglycemia in BM-reconstituted Id1+/-Id3-/- mice, but not in WT mice (Figure 5C-D). Control mice (i.e., not transplanted) remained diabetic over the study period (blood glucose >400 mg/dl, n=2, not shown). Furthermore, removal of the grafts at 3 weeks post-transplantation in cured mice led to recurrence of diabetes (blood glucose=283±47 mg/dl, mean ± SEM, n=8), confirming the functionality of the grafts and the lack of functional recovery of the endogenous islets from streptozotocin-mediated destruction. In addition, insulin immunostaining of pancreata revealed that remnant insulin+ areas were not significantly different in streptozotocyn-treated WT and Id1/Id3 deficient mice (not shown), indicating that there was no differential beta-cell recovery within the 4 weeks post-transplant period. Further experiments showed that serum insulin and adiponectin, and response to insulin challenge were similar in WT and Id1+/-Id3-/- mice (Online Appendix, Figure S5), indicating that differential peripheral insulin sensitivity did not account for the observed diabetes reversal by small islet transplants in BM-reconstituted Id1+/-Id3-/- mice.

These results demonstrate that the enhanced contribution of BM-derived cells to islet revascularization has a significant impact on transplant engraftment and function, allowing even a limited number of islets to reverse diabetes in transplant recipients.

DISCUSSION

BM-derived vascular cells have been proposed as targetable cell types for drug or gene delivery and for vascular repair (2). However, conditions permissive to the substantial recruitment of these cells in nontumoral tissues remain to be defined. Furthermore, it is uncertain whether such vasculature would support normal tissue functions. Here we show that functional BM-derived blood vessels can develop within pancreatic islet grafts and that Id1/Id3
defective expression at transplantation sites is required for this phenomenon. Neo-
vascularization by BM-derived vasculogenic cells is associated with enhanced islets’ vascular density and improved graft survival and function, demonstrating a biological advantage over the tissue-derived vasculature. Moreover, the down-regulation of genes involved in tissue damage and the activation of protective repairing responses observed in these grafted provide evidence for a role of BM-derived cells in antagonizing pathogenic inflammation and promoting tissue healing.

The vascular contribution of BM-derived vasculogenic cells vary greatly in tumors of different origin and grades, and in transplanted versus spontaneous tumors (9,20,22,35). These observations predict that recruitment and development of these progenitors may also differ among quiescent tissues. We demonstrate herein that islet transplants supports vascular development of BM-derived cells. Islets’ angiogenic factors such as VEGF (35) may contribute to this effect. Yet, these factors may not be sufficient, since the same tissue engrafted in WT mice harbored virtually no BM-derived blood vessels. Accordingly, previous reports have shown a few BM-derived vascular cells in models of islet transplantation or islet injury (37,38). In addition, over-expression of pro-angiogenic factors and/or use of progenitors mobilizing agents, while enhancing local accumulation of various BM-derived cell lineages, do not necessarily result in increased incorporation of BM-derived vascular cells into blood vessels (39,40). Thus, in a WT environment, the development of BM-derived vascular progenitors at sites of healing appears to be tightly regulated by unknown local homeostatic mechanisms. These tissue barriers are clearly overcome by down-regulation of Id1 and Id3 at transplantation sites, providing a niche for BM cells to home and/or expand. In this regard, it is noteworthy that Id1 in endothelial cells regulates expression of chondroitin sulfate proteoglycan and HIF1α, factors previously involved in the recruitment and/or development of BM-derived endothelial cells (41,42). Hence, down-regulation of Id1 and Id3 at transplantation sites (e.g. by retrovirus-mediated siRNAs delivered to the local vasculature) may be envisaged as a therapeutic strategy to facilitate homing/development of BM cells with high vasculogenic potential, thereby improving engraftment and function of cell transplants.

While our studies uncover distinct engrafting and angiogenic functions of BM-derived versus tissue-derived vasculogenic cells in islet transplants, they do not address to which extent these properties are contributed by the vascular cells and/or by associated inflammatory leukocytes. Indeed, vascular and inflammatory cells regulate each other during tissue healing (1). Further studies are warranted to determine how these complex cellular networks influence engraftment. Nevertheless, our gene screening experiments provide important clues on the cell types and molecular pathways possibly involved. Thus, a hallmark of the grafts supported by BM-derived blood vessels is the increased expression of genes regulating the influx, activation and angiogenic function of neutrophils and monocytes. Consistent with an increased frequency of these myeloid subsets, these grafts harbored a higher number of GR1highF480+ cells. In addition, fewer T cells were observed in those transplants. Interestingly, there is evidence that GR1+ leukocytes regulate the angiogenic switch in tumors (43) and that T cells control vascular pruning and remodeling (44). Hence, GR1highF480+ cells may contribute to the enhanced angiogenic response of the BM-derived vasculature, whereas the low number of T cells recruited and/or surviving locally may be permissive to the expansion of that vascular network in the islet grafts. The
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Increased expression of genes marking the activation of M2 polarized macrophages as well as anti-oxidative pathways point to other biological responses that may also positively affect engraftment. Importantly, this M2-polarized gene profile was not detected in peripheral macrophages (not shown), indicating that it was not dictated by the Id1/Id3-deficient environment \textit{per se}. This pattern provides strong evidence that BM-derived vascular cells are associated with, and/or may support, protective repairing rather than harmful inflammatory responses at sites of tissue injury. Finally, noteworthy for its direct implications on transplant survival, is the down-regulation of Factor D (adipsin) in the grafts from BM-reconstituted Id1+/−/Id3−/− mice. Adipsin is the limiting factor for the activation of the alternative pathway of complement, reportedly involved in ischemia/reperfusion injury (45). The down-regulation of this factor suggests that the microenvironment contributed by BM-derived vasculogenic cells modulates the susceptibility of islet tissue to damage by complement.

Islet transplantation has the potential to replace pancreatic endocrine function in Type 1 diabetics. However, the large β-cell mass required to treat individual patients has precluded the wide use of this approach. Our studies provide \textit{in vivo} evidence that neo-vascularization by BM-derived vasculogenic cells confers a significant survival advantage to islet transplants, allowing fewer islets to promptly reestablish normoglycemia in diabetic recipients. It will be important to determine in future studies whether BM-derived endothelial progenitors can similarly enhance islet engraftment at other transplantation sites (e.g. the liver) as well as in models of allo-transplantation in autoimmune diabetic mice. Notwithstanding, the syngeneic system presented here demonstrates that in the absence of a substantial input from this BM vasculogenic component, the angiogenic response of the tissue vasculature appears insufficient to ensure survival throughout the graft, as inferred from the heterogeneous pAkt expression observed \textit{in situ}. Notably, in the grafts of BM-reconstituted Id1+/−/Id3−/− mice, strong expression of nuclear pAkt inversely correlated with PML. This tumor suppressor gene opposes pAkt nuclear functions and negatively regulates responses to hypoxia and angiogenesis (26,46). Hence, PML down-regulation within islet grafts is consistent with a coordinated activation of survival and pro-angiogenic signals.

In conclusion, these studies provide strong evidence that enhancing the contribution of BM-derived vasculogenic cells is a promising therapeutic approach to improve post-transplant recovery of pancreatic islets. Furthermore, the association of BM-derived vascular cells with protective inflammatory responses shown here may have implications in transplant tolerance.

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| Inflammatory response genes                  | Accession Number | Whole Graft | CD31<sup>+</sup> cells | ISB4<sup>+</sup> cells | Function                                        |
|----------------------------------------------|------------------|-------------|-------------------------|------------------------|------------------------------------------------|
| Adiponectin                                  | NM_009605.3      | -2.6        | -1.2                    | 1.12                   | Anti-inflammatory, anti-angiogenic               |
| Leptin                                       | NM_008493.3      | -5.9        | 1                       | 1.1                    | Pro-inflammatory/ Th1 immune responses           |
| Resistin-like molecule alpha                 | NM_020509.2      | 24          | -3.1                    | 2.2                    | Pro-inflammatory/M2 polarized immune responses   |
| Interleukin 10                               | NM_010548.1      | 1           | 3.4                     | 1                      | Anti-inflammatory, Th2 immune responses          |
| TNFα                                         | NM_013693        | 1.4         | 2.7                     | 1                      | Th1 immune responses, pro angiogenic            |
| Interleukin 1β                               | NM_008361        | -7.1        | 1.6                     | 1.6                    | Th1 immune responses, pro-angiogenic            |
| Interleukin 4 induced 1                      | NM_010215.1      | 2.4         | -1.1                    | 1.2                    | Down-regulation of T cell responses             |
| CCL1                                         | NM_008510.2      | -2.0        | 1.9                     | 2.2                    | Eosinophils recruitment/ Th2/Tc2/Treg responses |
| CCL2 (MCP-1)                                 | NM_011333.1      | -1.3        | 2.6                     | 1.6                    | Monocyte recruitment/pro-angiogenic             |
| CCL5 (RANTES)                                | NM_013653.1      | -1.9        | 2.0                     | 1.7                    | Monocytes/T cells/eosinophils recruitment       |
| CCL7                                         | NM_013654        | -1.1        | 2.1                     | -1.5                   | Monocyte recruitment                            |
| CXCL9                                        | NM_008599        | -2.6        | 2.6                     | -1.4                   | Monocytes/T cell recruitment                    |

| Acute response/repair response genes          | Accession Number | Whole Graft | CD31<sup>+</sup> cells | ISB4<sup>+</sup> cells | Function                                        |
|----------------------------------------------|------------------|-------------|-------------------------|------------------------|------------------------------------------------|
| Complement Factor D (Adipsin)                | NM_013459.1      | -100        | 1.0                     | 1.0                    | Complement activation                            |
| Chitinase 3-Like 4                           | NM_145126.1      | 24          | -1.5                    | 2.9                    | Th2/M2 polarized immune responses               |
| Small proline-rich protein 2A                | NM_011468.2      | 7.6         | 1.0                     | 1.1                    | Protection from ischemic injury                 |
| Microsomal glutathione S-transferase 1       | NM_019946.3      | 5.7         | 1.0                     | -1.3                   | Protection from oxidant stress                  |
| Glutathione peroxidase 2                     | NM_030677.1      | 5.2         | -1.1                    | 2.9                    | Protection from oxidant stress                  |
| Interleukin 1 receptor, Type II              | NM_010555.2      | 1.2         | 1.4                     | 2.7                    | Decoy receptor, down-regulation of IL1 signaling |
| Trefoil Factor 1                             | NM_009362.1      | 1.2         | -1.2                    | 3.2                    | Anti-inflammatory, anti-angiogenic, pro-angiogenic |
| Trefoil Factor 2                             | NM_009363.2      | -2.4        | 4.7                     | 1.2                    | Anti-inflammatory, induction of cell proliferation/ migration |
| CCL24                                        | NM_019577.2      | 8.6         | -2.6                    | 1.8                    | Eosinophils recruitment/Th2/Tc2 responses        |
| SLAM member 7                                | AK089525         | -1.2        | 2.5                     | 1.1                    | Immune-regulation, Th2 responses, healing        |
| Scavenger receptor class A, member 3         | NM_172604.1      | -1.2        | 1.2                     | 4.4                    | Pathogen recognition, apoptotic cell clearance   |
| C-reactive protein (Pentraxin-related)        | NM_007678.2      | 1.2         | 1.1                     | 3.1                    | Pathogen recognition, apoptotic cell clearance   |
| Chitinase 3-like 1                           | NM_007695.1      | 1           | 3.0                     | 1.5                    | Down-regulation of IL1/TNF signaling            |
| Serine peptidase inhibitor (Clade G)         | NM_009776        | -1.8        | 2.0                     | 2.1                    | Complement Component 1 inhibition, suppression of leukocyte trans-migration |
| Serine peptidase inhibitor (Clade F)         | NM_011340.2      | -1.3        | 1.5                     | 2.4                    | Anti-inflammatory, anti-angiogenic, pro-angiogenic |
| Secretory Leukocyte Peptidase Inhibitor      | NM_011414.1      | -1.2        | 1.4                     | 5.7                    | Anti-proteases, anti-inflammatory                |
| Cathelicidin                                 | NM_009921.1      | 1           | 1                      | 13                     | Anti-microbial, immuno-modulatory                |
| Formyl peptide receptor like 1               | _NM_008391.1     | -3.7        | 2.4                     | 1.39                   | Neutrophils/monocyte/T cell recruitment, regulation of neutrophils life span, pro-angiogenic |

| Angiogenesis-related genes                    | Accession Number | Whole Graft | CD31<sup>+</sup> cells | ISB4<sup>+</sup> cells | Function                                        |
|----------------------------------------------|------------------|-------------|-------------------------|------------------------|------------------------------------------------|
| Calgranulin A                                | NM_013650.1      | -4.4        | 1.8                     | 6.5                    | Myeloid cells recruitment/pro-angiogenic        |
| Calgranulin B                                | NM_009114.1      | -5.4        | 1.5                     | 7.1                    | Myeloid cells recruitment/pro-angiogenic        |
| Coagulation factor X                         | NM_007972.1      | 1.2         | 1.0                     | 4.0                    | Coagulation Factor/pro-angiogenic               |
| Thy.1.2                                      | NM_009382.2      | 1.4         | -1.2                    | 4.2                    | Matrix interaction/pro-angiogenic               |
| Pro-collagen Type VI                         | NM_009933.1      | 2.8         | 1.8                     | 2.2                    | Expressed in tumor endothelium, ligand of TEM8   |
| Serum Amyloid 3                              | NM_011315        | -1.1        | 3.4                     | 3.3                    | Induction of MMPs/pro angiogenic                |
| Kruppel like factor 5                        | NM_009769.2      | -1.1        | 1.3                     | 3.2                    | Vascular remodeling                             |
| Interleukin-6                                | NM_031168.1      | 1           | 1.2                     | 3.2                    | VEGF induction/pro-angiogenic                   |
| Spondin 2                                    | NM_133903.2      | 1.3         | 1.2                     | 3.2                    | Cell spreading                                  |
| TIMP1                                        | NM_011593        | 1.3         | 1.5                     | 3.0                    | Regulation of MMPs/ECM remodeling               |
| MMP7                                         | NM_010810        | -2.2        | -1.6                    | 2.7                    | Endothelium proliferation/migration             |
| SPARC related protein                        | NM_022316.1      | -1.2        | 1.1                     | 2.6                    | Negative regulation of cell adhesion, ECM       |
| degradation                                  |                  |             |                         |                       |                                                 |
| Pro-collagen Type IV alpha3                  | NM_007734.1      | 1           | -1.3                    | -2.0                   | Blood vessels regression, anti-angiogenic       |
| Haptoglobin                                  | NM_017370.1      | -6.5        | -1.5                    | 2.7                    | Anti-oxidant, pro-angiogenic                    |
| Fas Ligand                                   | NM_010177.2      | -1.1        | 1                       | 3.1                    | Pro-angiogenic, pro-apoptotic                   |
| Interleukin 18 receptor                      | NM_008365.1      | 1.2         | 1.2                     | 2.8                    | Pro-inflammatory/pro-angiogenic                 |
| CXCR3                                        | NM_009910.1      | 0.8         | 1.9                     | 2.4                    | Endothelium proliferation/monocytes homing      |
| Mieloperoxydase                              | NM_010824        | 1.6         | -1.1                    | 2.2                    | Neutrophils phagocytic functions                |
| Cathepsin G                                  | NM_007800.1      | 0           | -1.2                    | 5                      | MMPs/receptors activation, pro-angiogenic       |
| Elastase 2                                   | NM_015779        | 0           | 1                       | 4.4                    | MMPs/receptors activation, pro-angiogenic       |
| Arginase I                                   | NM_007482        | -1.1        | -1.4                    | 6.4                    | Polyamine synthesis, cell proliferation         |
Table 1. Inflammation and angiogenesis-related genes differentially expressed in the grafts of BM-reconstituted Id1/Id3-deficient versus WT mice. Genes exhibiting at least a 2-fold change between Id1/Id3 deficient and WT mice in the indicated grafts’ cell fractions are displayed. Values equal to 1 indicate no change of gene expression in Id1/Id3 deficient versus WT mice, whereas positive and negative values indicate up-regulation and down-regulation of specific genes, respectively.
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FIGURE LEGENDS

Figure 1. Rescue of transplant engraftment in Id1+/-Id3-/- mice by reconstitution with WT BM is associated with the activation of islet cell survival signals (A) Grafts tissue sections at 1 week post-transplantation stained by immunoperoxidase for insulin (brown). Scar tissue occupies the transplantation site in untreated Id1+/-Id3-/- mice (middle panel, area bordered by dotted line) indicating failure of islet engraftment. In contrast, grafts containing insulin+ cells are present in BM-reconstituted WT and Id1+/-Id3-/- mice (upper and lower panels). (B) Morphometric analysis of whole grafts’ (upper graph) and insulin+ areas (lower graph) measured in multiple sections collected at 100 μm intervals throughout the grafts as described in Material and Methods. Each bar represents the mean ± SEM of measurements from 80-120 tissue sections per group with n=4 mice/group. (C) Quantitative analysis of apoptotic cells detected by TUNEL at 1 week post-transplantation. Values are mean ± SEM of measurements from n=3 mice per group. Statistical significance by t-test is indicated. Significance was also validated by ANOVA and Bonferroni posttest (Online Appendix). (D) Quantitative determination of pAkt[S473] (left) and total Akt (right) detected by ELISA in lysates of islet clusters microdissected from the grafts. Values are mean ± SEM of triplicate samples from a pool of n=2 grafts per group. (E) Tissue sections stained for pAkt[S473] by immunoperoxidase. In BM-reconstituted Id1+/-Id3-/- (upper panel) virtually all cells within islet cell clusters (dotted areas) express high levels of pAkt[S473] whereas in WT mice (lower panel) only cells at the periphery of the clusters (arrows) are strongly positive for pAkt[S473]. (F) Confocal microscopy of tissues sections stained by two color immunofluorescence for pAkt[S473] (red) and insulin (green). Most insulin+ cells are pAkt+ in BM-reconstituted Id1+/-Id3-/- mice (upper panel) whereas fewer insulin+ cells express pAkt in WT controls (lower panel). Inset represents background staining by control IgGs. Immunostainings are representative of n=3 grafts. (G) Tissue sections stained for PML by immunoperoxidase. Weak expression of PML in nuclea and cytoplasm of islet cells from the grafts of BM-reconstituted Id1+/-Id3-/- (upper panel) mirrors pAkt[S473] expression pattern. (H) Western blotting of PML and β-actin in protein lysates of islet clusters microdissected from the grafts demonstrates differential expression of PML in WT and Id1/Id3 deficient mice.

Figure 2. BM-derived vasculogenic cells form a dense network of functional blood vessels and differentially contribute to islet grafts revascularization in BM-reconstituted Id1+/-Id3-/- and WT mice. (A) Grafts tissue sections at 1 week post-transplantation stained by immunoperoxidase for PECAM-1, demonstrates a higher density of blood vessels in BM-reconstituted Id1+/-Id3-/- mice versus untreated Id1+/-Id3-/- and WT mice. Insets are enlarged areas of the displayed images. (B and C) Morphometric analysis of PECAM-1+ blood vessel density in whole grafts (B) and islets only (C), at 1 and 4 weeks post-transplant. In (C) the vascular density of islets endogenous to pancreata from WT mice is shown for comparison (brown bar at right). Islet tissue was identified by hematoxilin counterstaining and calculated areas verified by immunostaining for synaptophysin in consecutive sections. Values marked by * were derived from one surviving graft in a group of three, where the two other grafts failed, and were therefore unavailable for morphometric determination. Values are mean ± SEM of measurements from 80-120 tissue sections/ group. Statistical significance by t-test is indicated. Significance was also validated by ANOVA and Bonferroni posttest (Online Appendix). (D) Confocal microscopy of grafts tissue sections from mice at 4 weeks post-transplantation injected I.V. with FITC-ISB4 (green) to identify functional blood vessels, and stained by immunofluorescence for insulin (red). (E) To track the BM origin of vascular endothelial cells, Id1+/-Id3-/- and WT mice were reconstituted with ROSA26 BM, expressing β-gal in all nucleated cells. Labeling of blood vessels by ISB4 (green) and immunostaining of β-gal+ cells (red) demonstrates that grafts blood vessels in BM-reconstituted Id1+/-Id3-/- mice are of BM origin (arrows). In contrast, BM-derived β-gal+ endothelial cells are undetectable in the grafts of WT recipients. Images are representative of n=4 grafts.

Figure 3. Detection of inflammatory leukocytes in the islet grafts. (A) Tissue sections from islet grafts of BM-reconstituted WT and Id1+/-Id3-/- mice, at 1 and 4 weeks post-transplantation, stained by
two-color immuno-histochemistry for the pan-leukocyte marker CD45 (blue) and the myeloid marker F480 (brown), or control IgGs (inset). A leukocytic inflammatory infiltrate, comprising myeloid cells, is apparent in the grafts from both experimental groups. The dotted lines mark the border of the grafts with the kidney. The intense blue staining in the kidney is background due to color development by the alkaline phosphatase endogenous to the kidney epithelium. Images are representative of n=3 grafts per experimental group. (B) Flow cytometric analysis of leukocytes isolated from the grafts at 1 and 4 weeks post-transplantation stained by two color immunofluorescence for the myeloid markers GR1 and F480. An increased percentage of GR1$^{\text{high}}$F480$^{+}$ cells in the graft of BM-reconstituted Id1+/−Id3−/− mice is evident as compared to WT controls. Theses cells are not present in the bone marrow of either mice. The dot plots are representative of n=4 experiments. (C and D) Quantitative analysis of GR1$^{\text{high}}$F480$^{+}$ and CD3$^{+}$ cell subsets detected by flow cytometry in the grafts BM-reconstituted Id1+/−Id3−/− and WT mice at 1 and 4 weeks post-transplantation. Bars are mean ± SEM of n=4 independent determinations.

Figure 4. Heatmaps of genes differentially expressed in whole grafts and cellular fractions of BM-reconstituted Id1+/−Id3−/− mice over WT controls. Genes were grouped as inflammatory, repair and angiogenesis-related genes. Fold changes between gene expression levels in samples obtained from BM-reconstituted Id1+/−Id3−/− mice over those of WT controls are presented in the form of a heatmap (blue to red scale). Only genes displaying >2 fold changes are shown.

Figure 5. Functionality of islet grafts in diabetic recipients. (A) Blood glucose levels in Id1+/−Id3−/− and WT Id1+/+Id3+/+ mice following streptozotocin injection. Shadowed area mark the limit of blood glucose values above which mice were considered frankly diabetic. (B, C and D) Levels of blood glucose in mice rendered diabetic by streptozotocin, and 1 week later transplanted with 500 (B), 200 (C) or 75 (D) WT islets per mouse. Values are mean ± SEM of measurements from the indicated number of mice.
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**Figure 2**

**A**

WT + WT BM

ld1+/-ld3+/

ld1+/-ld3/- + WT BM

**B**

Blood Vessels Density (% of grafts area)

p = 0.009

**C**

Blood Vessels Density (% of grafts area)

p = 0.62

p < 0.01

p < 0.001

**D**

In insulin / Isolectin B4

WT + WT BM

ld1+/-ld3+/

ld1+/-ld3/- + WT BM

**E**

anti-β-gal Ab

Isolectin B4

merge

Graft

Kidney

Graft

Kidney

Miller et al., Figure 2
Islets engraftment by BM-derived Vasculogenic Cells

**A**

| 1 week | 4 weeks |
|--------|---------|
| WT + WT BM |  |  |
| Id1+/-Id3-/- + WT BM |  |  |

**B**

| Bone Marrow | Transplants |
|-------------|-------------|
| 1 week | 4 weeks |
| WT + WT BM |  |  |
| Id1+/-Id3-/- + WT BM |  |  |

**C**

|  | WT + WT BM | Id1+/-Id3-/- + WT BM |
|---|------------|-----------------------|
| Total F480+ cells |  |  |
| GR1+/F480+ cells |  |  |

**D**

|  | GR1+/F480+ cells | CD3+ cells |
|---|------------------|------------|
| Weeks post-transplantation | 1 | 4 |
| 1 |  |  |
| 4 |  |  |

Miller et al., Figure 3
**Islets engraftment by BM-derived Vasculogenic Cells**

### Inflammatory response

| NCBI Acc | Description |
|----------|-------------|
| MM_02099.2 | resltnl lite alpha (Histil) |
| MM_01048.1 | interleukin 10 (IL10) |
| MM_01054.1 | chemokine (C-C motif) ligand 7 (CCL7) |
| MM_01133.1 | chemokine (C-C motif) ligand 2 (CCL2) |
| MM_01493.1 | tumor necrosis factor (TNF) |
| MM_01493.1 | chemokine (C-C motif) ligand 5 (CCL5) |
| MM_00851.0 | chemokine (C motif) ligand 1 (CXCL1) |
| MM_00859.1 | chemokine (C-X-C motif) ligand 9 (CXCL9) |
| MM_00843.3 | interleukin 1 beta (IL1B) |
| MM_00493.3 | leptin (Lep) |
| MM_00940.5 | adipocyte, C1Q and collagen domain containing (Acod) |

### Acute/reparatory response

| NCBI Acc | Description |
|----------|-------------|
| MM_01377.2 | chemokine (C-C motif) ligand 24 (CCL24) |
| MM_01024.1 | chitinase 3-like 1 (Chi3l4) |
| MM_01346.1 | microosomal glutathione S-transferase 1 (Gstpi) |
| MM_01468.1 | small proline-rich protein 4A (Sprz2a) |
| MM_00777.1 | glutathione peroxidase 2 (GPx2) |
| MM_00913.1 | urocortin 2 (Ucn2) |
| MM_01414.1 | secretory leukocyte protease inhibitor (SLPI) |
| MM_01704.1 | scavenger receptor class A, member 3 (Scara3) |
| MM_00976.1 | serine (or cysteine) proteinase inhibitor, clade G, member 1 (serping1) |
| MM_01346.1 | serine (or cysteine) proteinase inhibitor, clade F, member 1 (serping1) |
| MM_00962.1 | trefoil factor 1 (TFF1) |
| MM_00768.2 | C-reactive protein, pentaxin related (Crp) |
| MM_01867.1 | C1s, k, e, and f, collagenase, type VI (C1s60) |
| MM_01009.1 | interleukin 1 receptor, type II (IL1r2) |
| MM_00785.1 | chitinase 3-like 1 (Chi3l1) |
| MM_00936.3 | trefoil factor 2 (tff2) |
| MM_00639.1 | formyl peptide receptor, related sequence 2 (Fpr2r2) |
| MM_01345.1 | agouti (Agout) |

### Angiogenesis

| NCBI Acc | Description |
|----------|-------------|
| MM_01308.1 | P-box and WD-40 domain protein 5 (Pw5) |
| MM_01024.1 | myeloperoxidase (Mpo) |
| MM_00822.2 | lactotransferrin (LTF) |
| MM_00923.1 | procollagen, type V, alpha 1 (Covta1) |
| MM_01195.1 | tissue inhibitor of metalloproteinases 1 (Timp1) |
| MM_00769.1 | Kruppel-like factor 1 (KLF1) |
| MM_01316.1 | interleukin 6 (Il6) |
| MM_00309.1 | apolipoprotein A, secretory leucine rich protein (Lpaa) |
| MM_00855.1 | interleukin 18 receptor 1 (IL18r1) |
| MM_00772.1 | caspase 3 (CASP3) |
| MM_00989.1 | angiotensinogen (Ang) |
| MM_01197.1 | neutrophil elastase (Neu) |
| MM_01077.1 | tumor necrosis factor (ligand) superfamily, member 6 (Tnfαf6) |
| MM_00740.1 | heme oxygenase 1 (Hmox1) |
| MM_00910.1 | secreted frizzled-related protein 2 (Sfrp2) |
| MM_00774.1 | procollagen, type IV, alpha 3 (Cov4a3) |

*Miller et al., Figure 4*
Islets engraftment by BM-derived Vasculogenic Cells

Miller et al., Figure 5