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RESEARCH PAPER

Direct comparison of the abilities of bone marrow mesenchymal versus hematopoietic stem cells to reverse hyperglycemia in diabetic NOD.SCID mice

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ABSTRACT

Both bone marrow-derived hematopoietic stem cells (HSC) and mesenchymal stem cells (MSC) improve glycemic control in diabetic mice, but their kinetics and associated changes in pancreatic morphology have not been directly compared. Our goal was to examine the time course of improvements in glucose tolerance and associated changes in β-cell mass and proliferation following transplantation of equivalent numbers of HSC or MSC from the same bone marrow into diabetic non-obese diabetic severe combined immune deficiency (NOD.SCID) mice. We used transgenic mice with a targeted expression of yellow fluorescent protein (YFP) driven by the *Vav1* gene promoter to genetically tag HSC and progeny. HSC were separated from bone marrow by fluorescence-activated cell sorting and MSC following cell culture. Equivalent numbers of isolated HSC or MSC were transplanted directly into the pancreas of NOD.SCID mice previously made diabetic with streptozotocin. Glucose tolerance, serum insulin, β-cell mass and β-cell proliferation were examined up to 28 days following transplant. Transplantation with MSC improved glucose tolerance within 7 days and serum insulin levels increased, but with no increase in β-cell mass. Mice transplanted with HSC showed improved glucose tolerance only after 3 weeks associated with increased β-cell proliferation and mass. We conclude that single injections of either MSC or HSC transiently improved glycemic control in diabetic NOD.SCID mice, but with different time courses. However, only HSC infiltrated the islets and were associated with an expanded β-cell mass. This suggests that MSC and HSC have differing mechanisms of action.

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Bone marrow stem cells; diabetes; HSC; mouse; MSC; pancreas; plasticity; regeneration; β-cell

Introduction

Bone marrow stem cell (BMSC) transplants cause at least a temporary reversal of experimental diabetes in rodents, and possibly also in human type 1 diabetes.[1-4] Trans-differentiation of BMSC directly into pancreatic β-cells may occur for a small percentage of cells,[2,4] but the reversal of hyperglycemia appear to be mainly indirect through either increased function and/or proliferation in remaining β-cells,[5-7] or suppression of immune responses in the damaged pancreas to allow endogenous regeneration to proceed.[8] BMSC express the CXCR4 chemokine receptor[9] allowing them to home quickly to the diabetic pancreas in response to chemokines such as SDF-1 released from islets during pancreatic damage.[10] However, the fractions of BMSC responsible for reversing hyperglycemia, and their respective mechanisms, are poorly understood.

The two major BMSC lineages exist, hematopoietic stem cells (HSC) and mesenchymal stem cells (MSC), and both may contribute to β-cell regeneration. HSC give rise to all myeloid and lymphoid cell lineages, including endothelial precursor cells that can contribute to microvascular angiogenesis.[11] The contribution to angiogenesis is potentially important to β-cell regeneration since pancreatic vascular endothelium can induce β-cell differentiation from progenitors, while also providing key signals, such as hepatocyte growth factor (HGF), to promote β-cell proliferation and function.[12,13] When the ontogeny of HSC and their progeny genetically tagged with yellow fluorescent protein (YFP) was tracked in the young streptozotocin (STZ)-diabetic mouse there was an

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increased localization of HSC to the damaged pancreas preceding a regeneration of ß-cell mass.[14] HSC-derived cells expressing endothelial markers were found within and around the islets of Langerhans and adjacent to sites of endocrine cell neogenesis within the pancreatic ducts.

MSC transplantation into diabetic rodents causes a rapid reversal of hyperglycemia and may also improve islet vascularization and β-cell mass,[15-17] although the latter finding has been inconsistent.[18] Human exocrine pancreas is a source of endogenous MSC, and when isolated and expanded *in vitro* these cells developed endocrine progenitor phenotypic markers such as Pdx1 and Ngn3 expression, but not insulin.[19] As with HSC, green fluorescent protein (GFP)-labeled MSC migrate into the pancreas following STZ-induced diabetes in mice, but their distribution differs from HSC, being mainly in the exocrine pancreas rather than the islets.[20]

Thus, while endogenous HSC and MSC each mobilize to the damaged pancreas in the presence of hyperglycemia neither appears to be a substantial direct source of new β-cells, and they distribute to different tissue compartments. Comparison of the efficacy of BMSC-derived HSC or MSC in reversing diabetes has been complicated by the use of differing transplanted cell number, methods of delivery, and models of rodent diabetes. In order to directly compare their efficacy in reversing hyperglycemia, and any associated changes in pancreatic morphology, we have utilized in this study equivalent numbers of MSC or HSC isolated from the same bone marrow and transplanted directly into the pancreas of immune-deficient diabetic mice.

Materials and methods

Animals

Mice were housed in pathogen-free surroundings with a 12:12-h light-dark cycle in the animal facility, Lawson Health Research Institute, London, Ontario. Animals received food and water ad libitum. *Vav1* promoter-Cre mice (B6.Cg-Tg(Vav1-cre)A2Kio/J)[21] were purchased from Jackson Laboratories (Sacramento, CA, USA) as were ROSA26-YFP mice (B6.129X1-Gt(ROSA)26Sortm1(EYFP)Cos/J), and crossed to generate double transgenic Vav-Cre/YFP mice with YFP expression targeted to HSC and their progeny. Double transgenic offspring (Vav-Cre; R26R-EYFP) were identified by polymerase chain reaction of genomic DNA using primers: iCre sequence 5'-AGA TGC CAG GAC ATC AGG AAC CTG; 5'-ATC AGC CAC ACC AGA CAC AGA GAT C; and R26R-EYFP sequences oIMR0316 5'-GGA GCG GGA GAA ATG GAT ATG; oIMR0883 5'-AAA GTC GCT CTG AGT TGT TAT; oIMR4982 5'-AAG ACC GCG AAG AGT TTG TC, as described by us previously.[14] NOD.SCID mice (NOD.CB17-Prkdc^{SCID}/NcrCrl) were obtained from Charles River Laboratories (Sherbrooke, ON, Canada) and served as recipients of HSC or MSC transplantation.

Fluorescent activated cell sorting

Three month-old Vav1-Cre/YFP male mice were euthanized, the femur and tibia dissected, and bone marrow flushed into a sterile Petri dish containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 units/ml penicillin, fetal bovine serum (10 % v/v), streptomycin (100 µg/ml), L-glutamine (4 mM) and sodium pyruvate (1 mM). Dispersed marrow cells were centrifuged at 1200 rpm for 4 min at 4°C. The supernatant was aspirated and the pellet re-suspended in 1 ml red blood cell lysis buffer (Sigma-Aldrich, Oakville, ON, Canada) and incubated at 4°C for 5 min. Dulbecco's modified Eagle's (DME)/F12 medium (5 ml) and 10% v/v heat-inactivated FBS were added to each tube and the cells dispersed and filtered through a sterile 40 µm filter. Cell suspensions from YFP-expressing mice animals were pooled, as were those from controls. YFP$^-$expressing or non-expressing cells (300 µl) + 40 µl 7-aminoactinomycin D (7-AAD) as a cell viability indicator were subjected to FACS, as described previously.[22]

Isolation and propagation of bone marrow MSC

Bone marrow fractions were prepared from the same male Vav1-Cre/YFP transgenic mice as described above to isolate MSC using a previously published protocol.[23] Bone marrow was transferred into ventilated cap T-75 tissue culture flasks containing 10 mL DMEM and incubated in a humidified incubator at 37°C with 5 % CO_2.

Second passage MSC were used for transplant. Phenotypic identity of MSC was confirmed using flow cytometry for the presence of CD44, CD90 and CD117, as described by us previously.[24]

Experimental diabetes

Female NOD.SCID mice from each litter received either a subcutaneous injection of 35mg/kg STZ (Sigma-Aldrich) freshly prepared in citrate buffer (pH 4.5), or a sham injection consisting of citrate buffer alone, at 2 and 5 days of age. The latter animals served as controls. Some animals were sacrificed 72h following the last treatment with STZ to confirm destruction of the β-cells by immunohistochemistry of the pancreas. A non-fasted random blood glucose was measured using a glucometer (Accu-Check, Roche) 14 days after the last STZ injection to confirm the presence of diabetes (glucose >11 mM).

Intra-pancreatic cell transplant

As BMSC administered intravenously become lodged in the lungs and spleen with only a small proportion reaching the target organ,[25] we performed direct intra-pancreatic grafting as described previously by Bell *et al.*[26] MSC or HSC derived from Vav1-Cre/YFP male mice were transplanted into the pancreata of 20 days old, STZ-treated or control NOD.SCID female mice. Recipient mice were anaesthetized with isofluorane and a laparotomy was performed under sterile conditions to expose the spleen and the pancreas. HSC or MSC were microinjected (1×10^6 cells suspended in 150µl PBS) into the splenic portion of pancreas with a 25 G needle. Control animals received identical surgical intervention but were given a sham intra-pancreatic injection of PBS only.

Glucose tolerance test and insulin measurement

An intraperitoneal glucose tolerance test (IPGTT) was performed at days 7, 21 and 28 after HSC or MSC transplant into NOD.SCID mice, immediately before sacrifice. A bolus of 2g/kg glucose was injected in the peritoneal space (i.p.) and blood glucose monitored at 5, 15, 30, 60, 90 and 120 min. Glucose was measured by glucometer after lancing the tail vein. Blood was collected at sacrifice immediately after the glucose measurement at 120 min and serum insulin measured using an Ultrasensitive Insulin ELISA (range 0.02-1 ng/ml) (Crystal Chem., IL, USA).

Immunohistochemistry

NOD.SCID mice transplanted with HSC or MSC were sacrificed at 7, 21 or 28 days following transplant. The pancreas was removed and weighed, fixed in 4% paraformaldehyde, and embedding in OCT (Tissue-Tek, VWR International, Mississauga, ON, Canada). Cryosections (7 µm thickness) were placed on SuperFrost Plus glass slides (Fisher Scientific, Toronto, ON, Canada) and stored at −80°C with desiccant in an airtight box. Transplanted HSC and their progeny were identified by fluorescence microscopy for YFP. To determine the presence of the male transplanted MSC within the pancreas of female recipient mice, detection of the Y-chromosome was performed using the Mouse IDetect Y chromosome fluorescence *in situ* hybridization (FISH) kit (Empire Genomics, Buffalo, NY, USA).

Fluorescence immunohistochemistry co-staining was performed for insulin and Ki67 to detect co-localization of DNA synthesis in β-cells, and for CD31 and insulin to calculate the density of endothelial cells per islet. Non-specific binding was prevented with blocking solution (Sniper Universal block; Biocare Medical, Concord, CA, USA) before incubation with rabbit anti-GFP (1:200; Abcam, Toronto, ON, Canada) for YFP detection, mouse or rabbit anti-insulin (1:2000, Sigma Chemical Co., St Louis, MO; and 1:300, Santa Cruz respectively), mouse anti-Ki67 (1:50 BD Biosciences, Mississauga, ON, Canada), or a monoclonal antibody against CD31 (1:50, Hycult Biotech, Plymouth Meeting, PA, USA) overnight in a humidified chamber at 4°C. After washing with PBS, secondary antibodies were applied (1:400 donkey anti-goat or anti-rabbit, AlexaFluor 488 and 555; or 1:400 goat anti-mouse, Alexafluor 555 (Invitrogen Canada Inc., Burlington, ON, Canada) for 1h at room temperature. Nuclei were counterstained using 4, diamidino-2-phenylindole (1:1000; DAPI; 5 µg/ml) before mounting with coverslips using Dako fluorescent mounting medium (Dako, Mississauga, ON, Canada). To establish

specificity of the antibodies controls included substitution of the primary antibody with non-immune serum, or omission of the secondary antibody.

To detect β-cell mass, immunohistochemistry for insulin was performed using a modified avidin-biotin peroxidase method as described previously.[27] Following incubation with rabbit anti-insulin sections were incubated with biotinylated horse anti-mouse secondary antibody (Vector Laboratories, Burlington, Canada), and diaminobenzoate (DAB) (Biogenex Inc, Fremont, CA) was used as the chromogen. Sections were counter-stained with Carazzi's haematoxylin and morphometric analysis performed by light microscopy.

Morphometric analysis

Histomorphometry was performed using a Carl Zeiss Axioskop fluorescent microscope (Carl Zeiss, Inc., New York, NY, USA) with QImaging MicroPublisher 3.3 Real Time Viewing camera (QImaging, Burnaby, BC, Canada). Image analysis was performed using Northern Eclipse Version 7.0 morphometric analysis software (Empix Imaging, Inc., Mississauga, ON, Canada). For each section the following were determined: the total area of pancreatic tissue, the area of the pancreatic tissue represented by endocrine cells, the percentage of area occupied by β-cells (insulin-positive) within each islet, the percentage of insulin-positive cells that co-stained for Ki67, and the number of CD31-positive cells per mm^2 of islet.

Statistical analysis

Data is presented as mean ± SEM from between 4 and 12 animals per experimental group. Statistical analysis was performed using two-way ANOVA and Bonferroni's post hoc test for multiple comparisons, or by t-test. A value of $p<0.05$ was considered statistically significant. For morphometric analysis, three random sections (separated at least for 150μm) from each pancreas were analyzed. The β-cell mass per pancreas was calculated by multiplying the area of pancreas occupied by β-cells by the pancreatic weight. For statistical analyses the Prism software package (Graphpad Software Inc, La Jolla, CA) was utilized.

Results

Effects of STZ with or without cell transplants on body weight and fasting glucose

The mean values for body weight and fasting blood glucose 7, 21 and 28 days following sham or pancreatic transplants of HSC or MSC in NOD.SCID STZ-treated mice compared to control animals are shown in Table 1. Body weight did not significantly differ with time after treatment, or between treatments groups. Mice receiving STZ alone became significantly hyperglycemic compared to controls at days 21 and 28. Fasting blood glucose remained elevated in STZ-treated mice transplanted with MSC but was significantly reduced compared with STZ treatment alone in HSC-transplanted animals at day 21. However, neither MSC or HSC transplant were able to prevent hyperglycemia 28 days after transplant

Changes in glucose tolerance and circulating insulin

We examined if intra-pancreatic transplant of either HSC or MSC from Vav1-Cre/YFP mice could improve either glycemic control following an IPGTT or circulating insulin in STZ-treated NOD.SCID animals. IPGTTs were performed at 7, 21 and 28 days following cell transplantation compared with STZ-treated NOD.SCID sham-injected control animals, and the area under the curve (AUC) calculated. Mice given STZ but no

Table 1. Changes in fasted blood glucose and body weight at 7, 21 and 28 days following transplant of HSC or MSC into STZ-diabetic NOD.SCID mice compared to non-diabetic control animals.

Time post-transplant (days)	Treatment	Glucose (mmol/l)	Body weight (g)
7	Control	6.3 ± 0.8	14.8 ± 1.1
	STZ	9.9 ± 1.9	11.6 ± 0.9
	HSC	13.5 ± 1.9	13.5 ± 1.6
	MSC	9.2 ± 1.8	14.7 ± 2.4
21	Control	5.4 ± 0.5	20.2 ± 0.8
	STZ	22.1 ± 2.8	15.7 ± 0.6
	HSC	7.2 ± 0.7*	19.4 ± 0.9
	MSC	16.5 ± 3.5	19.2 ± 0.8
28	Control	5.9 ± 0.2	21.5 ± 1.2
	STZ	24.7 ± 3.3	17.5 ± 1.0
	HSC	28.2 ± 5.2	17.3 ± 1.2
	MSC	24.8 ± 6.3	14.4 ± 1.2

Values show ± SEM, * $p<0.05$ vs STZ at day 21 (One way ANOVA, Bonferroni post-hoc test). n = 4–10 animals.

cell transplant experienced severe glucose intolerance following an IPGTT at all time points (Fig. 1). Mice treated with STZ which received MSC, but not those transplanted with HSC, showed significantly better glucose control with a lower AUC compared to non-transplanted, STZ-treated NOD.SCID controls. At 21 days, both MSC and HSC transplants resulted in a significantly lower AUC compared to STZ-treated animals However, 28 days after pancreatic transplant all beneficial effects of HSC or MSC on glycemic control were lost. Serum insulin values were significantly reduced following STZ treatment at days 7 and 21 but returned to control values in mice transplanted with MSC, but not HSC at day 21 (Fig. 2). However, the increased insulin levels seen with MSC were not maintained at 28 days at which time they were similar to STZ-treated, sham-operated mice. Thus, MSC had a rapid beneficial effect on glucose tolerance at day 7 and 21 in STZ-treated mice, with a significant increase in circulating insulin at day 21. Conversely, HSC only improved glucose tolerance after three weeks although circulating insulin was still reduced compared to sham-operated controls. The positive effects of either MSC or HSC on glucose control were not sustained at 28 days.

Changes in pancreatic morphology

Despite the beneficial effects of MSC transplant on glycemic control, when the presence of transplanted MSC from male donors within the female pancreas was examined by FISH at day 7–28 they were not detectable. In contrast, transplanted YFP-expressing HSC were still detectable within the pancreas, and particularly associated with islets, at 21 days but much less so at 28 days (Fig. 3). No histological evidence of pancreatitis was observed as a result of the direct pancreas injection.

Beta-cell mass was calculated following immunostaining for insulin, as shown in representative images for control, STZ-treated, and stem cell transplanted animals in Fig. 4. No significant differences in β-cell mass were observed 7 days after transplant of MSC or HSC compared to sham-operated STZ-treated NOD.SCID controls. At day 21 STZ-treated NOD.SCID mice transplanted with HSC had a significant increase in β-cell mass compared to STZ-treated,

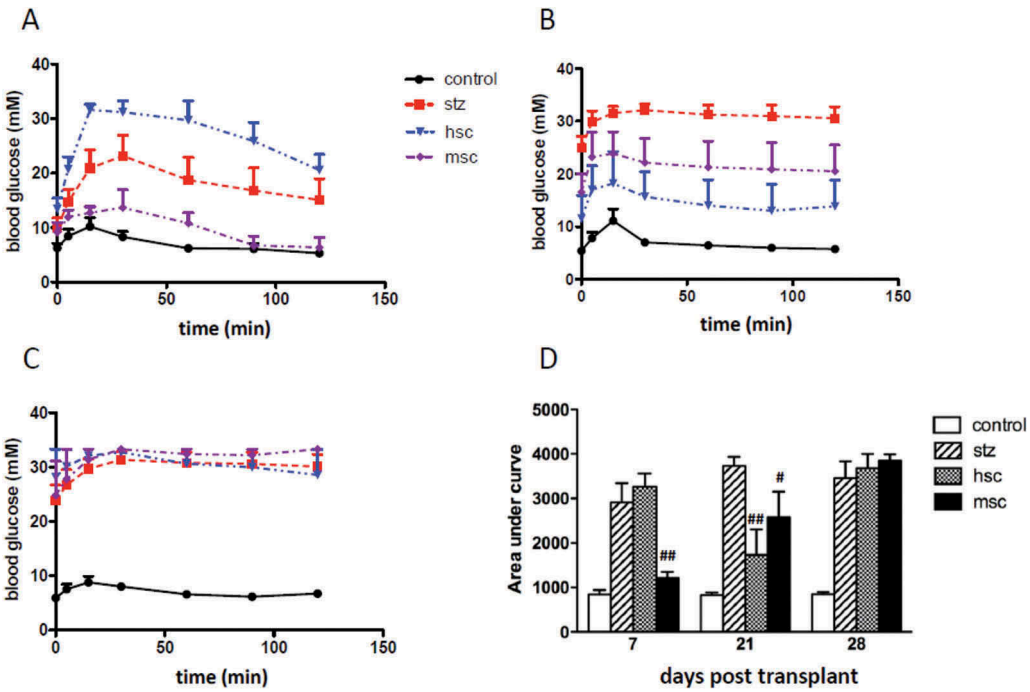

Figure 1. Glucose tolerance tests were performed on control, citrate buffer-treated mice (sham transplant, ●, open bar), mice treated with STZ (sham transplant, ■, hatched bar), and those who received STZ followed by intra-pancreatic transplant of HSC (▼, stipple bar) or MSC (◆, closed bar) at 7 (A), 21 (B) or 28 (C) days post-grafting. The areas under the curve are shown in D. Values show mean ± SEM; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ *vs.* STZ alone; n = 4–12 animals per time point.

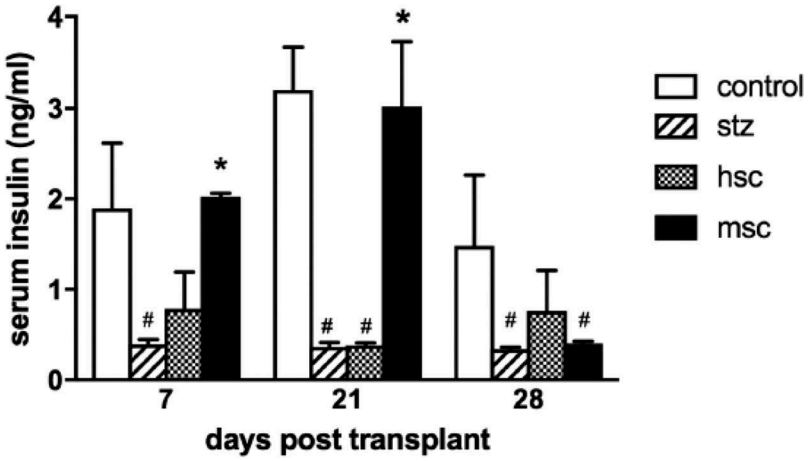

Figure 2. Serum insulin levels in control (citrate buffer-treated, sham transplant, open bar), mice treated with STZ (sham transplant, hatched bar), and those who received STZ followed by intra-pancreatic transplant of MSC (stippled bar) or HSC (solid bar) at 7, 21, or 28 days post-transplant. Values show mean ± SEM; # $p<0.01$ *vs*. control, * $p < 0.05$ *vs*. STZ alone for same time point; n = 4–8 animals per time point.

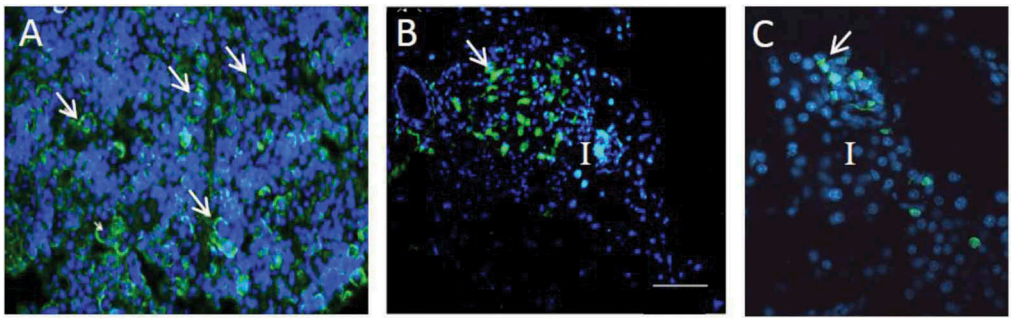

Figure 3. A) Presence of transplanted HSC from Vav1-Cre transgenic mice expressing YFP within the spleen of donor animals as a positive control. The arrows indicate YFP-expressing splenocytes. HSC were also located within the pancreatic islets (I) of recipient NOD.SCID mice 21 days (B) or 28 days (C) after transplantation. In each case the arrows indicate YFP-expressing HSC-derived cells. Cell nuclei are visualized using DAPI stain (blue). The magnification bar indicates 100 μm.

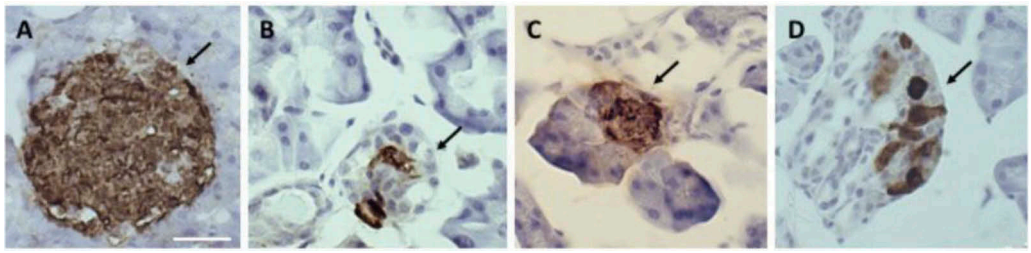

Figure 4. Immunohistochemical localization of insulin (brown) in β-cells within representative islets of Langerhans (arrows) within pancreata of control mice (A), mice treated with STZ (B), STZ with subsequent transplant of HSC (C) or STZ with subsequent transfer of MSC (D). The magnification bar indicates 100 μm.

sham-operated controls, such that the β-cell mass was equal to that of non-STZ-treated animals (Fig. 5). However, this relative gain in β-cell mass was not maintained at 28 days. To determine if this represented a subsequent loss of β-cells we measured the mean islet area occupied by insulin immunoreactive cells. For mice transplanted with HSC the mean ± SEM area of an islet at day 21 was 1590 ±292 μm^2 of which 88% was occupied by insulin-positive cells, whilst at day 28 the mean area of an

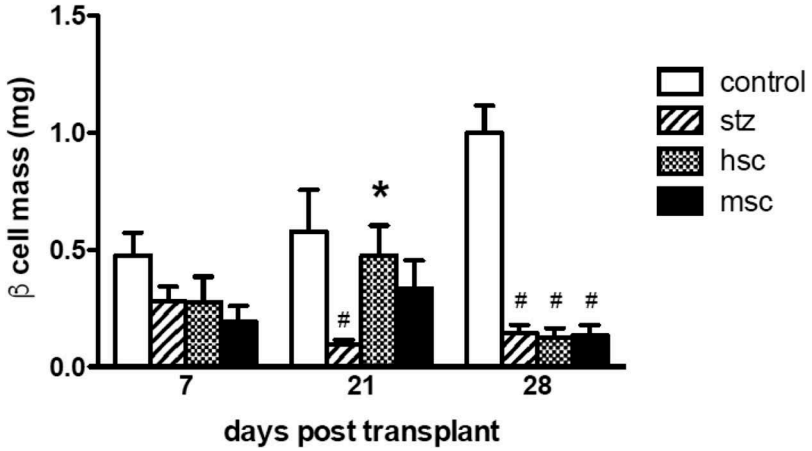

Figure 5. Pancreatic β-cell mass in control (citrate-buffer injected, sham transplant, open bar), mice treated with STZ (sham transplant, hatched bar), and those who received STZ followed by intra-pancreatic transplant of HSC (stippled bar) or MSC (closed bar) at 7, 21 or 28 days post-transplant. Values show mean ± SEM; $^{\#}$ $p < 0.05$ *vs.* control, * $p < 0.05$ *vs.* STZ alone, n = 4–11 animals per time point.

islet was 1631±92 μm^2 but with only 62% being occupied by insulin-positive cells. The maintenance of mean islet size suggests that at 28 days many β-cells may have become exhausted of insulin and no longer demonstrated insulin immunoreactivity. No significant changes were seen in β-cell mass in mice transplanted with MSC cells at any time point.

We next examined the relationship across all experimental groups between β-cell mass at time of sacrifice and the AUC during an IPGTT (Fig. 6). No significant relationship (R^2 = 0.11) existed at 7 days post-HSC or MSC transplant, despite the beneficial effects of MSC on glycemic control. However, at day 21 an inverse relationship existed between AUC and log β-cell mass with a slope significantly different from zero (R^2 = 0.65, $p<0.001$). This was also seen at day 28 (R^2 = 0.52, $p<0.05$). This analysis indicated that glycemic control was strongly dependent on the ß-cell mass of the animal, but that the ability of MSC to improve glycemic control at day 7 was not related to β-cell mass.

To determine the cellular compartments within the pancreas that contributed to an increased β-cell mass at day 21 after HSC transplant we examined the number of islets per area of pancreas, and the number of small, extra-islet endocrine cell clusters. The number of islets was increased compared to the NOD.SCID STZ-treated control mice on day 21, but not so the number of smaller endocrine clusters (Fig. 7A and B). No changes in islet or cluster number were seen in NOD. SCID STZ-treated mice after transplant of MSC. To determine if the increase in ß-cell mass at day 21 after HSC transplant was due to an increased cell proliferation, Ki67 was co-localized within β-cells and the abundance of Ki67-positive cells was found to be significantly increased compared to STZ-treatment alone at 21 days post-transplant, but not at 28 days (Fig. 7C). Beta-cell proliferation in both islets and clusters contributed to the increase on day 21.

Since the ability of transplanted HSC to increase β-cell mass following STZ treatment has been shown previously to be associated with their trans-differentiation into cells expressing endothelial progenitor cell markers,[2] we examined the density of vascular endothelial cells within islets from control and STZ treated mice, with and without transplant of MSC or HSC, 21 days following transplantation. CD31-immunoreactive cells were identified within control islets (Fig. 8). Following STZ the β-cell mass was substantially reduced, as were the number of CD31-positive cells per islet area. However, when quantified, the number of endothelial cells per mm^2 of islet was not significantly different from non-transplanted animals (Table 2).

The results show that HSC transplant was associated with an increase in β-cell mass, proliferation, and number of islets, and improved glucose tolerance after three weeks. In contrast MSC transplant resulted in an early improvement in glycemic

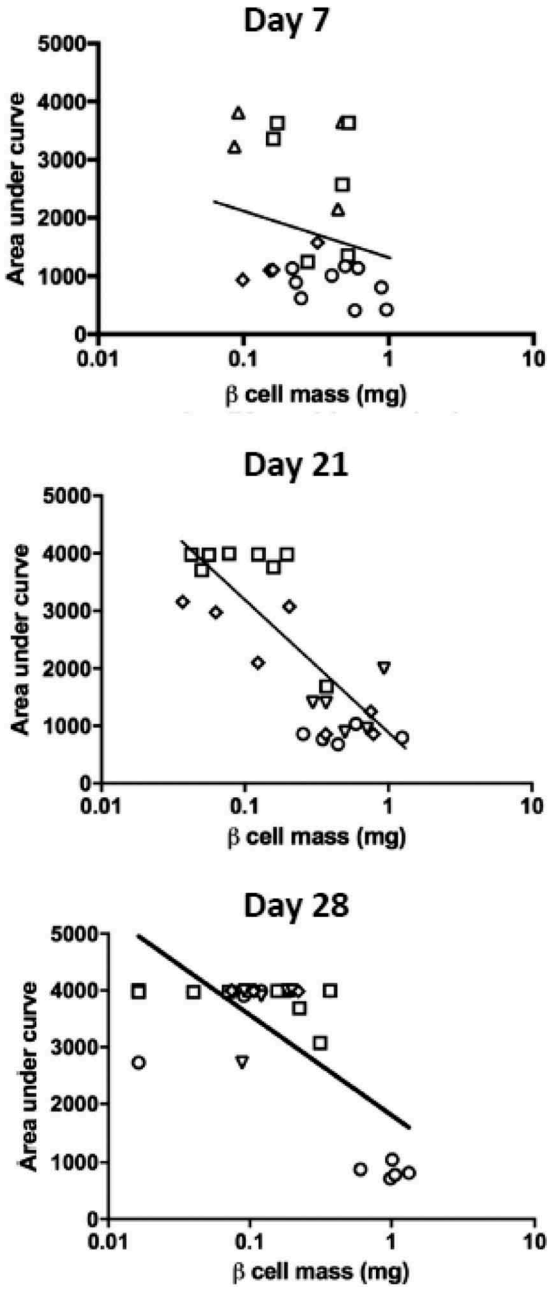

Figure 6. Correlation between the AUC following an IPGTT and β-cell mass at 7, 21 and 28 days following transplant of MSC or HSC. Control mice are represented by circles, STZ-treated mice by squares, STZ+MSC by diamonds, and STZ+HSC by triangles. The line of best fit is shown (day 7 $R^2 = 0.11$, day 21 $R^2 = 0.65$, day 28 $R^2 = 0.52$).

control, but no change in β-cell mass or islet morphology.

Discussion

This study provided a direct comparison of HSC and MSC derived from the same bone marrow for their ability to reverse diabetic hyperglycemia and the association with β-cell mass. Whilst both were effective at improving glucose tolerance, there were substantial differences in both time course and resulting changes in β-cell recovery.

Although transplanted YFP-expressing HSC or progeny could be detected in the pancreas up to 21 days post-transplant, no MSC remained detectable by FISH after 7 days. This agrees with previous reports that MSC transplanted into diabetic rats were detectable in pancreas for 7–8 days, but not thereafter.[28,29] This may be explained by the peak release of SDF-1 from injured pancreas 5–7 days after the insult, responsible for activating the CXCR4 receptor on MSC to promote migration.[29]

Despite a rapid clearance from the pancreas, transplanted MSC were able to reverse diabetic hyperglycemia after 7 days and the effect persisted until 21 days post-transplant, accompanied by a significant increase in serum insulin levels. However, no significant regeneration of β-cell mass or change in the number of islets or small pancreatic endocrine clusters occurred compared to non-transplanted mice. This implies a rapid impact of MSC on the functionality of remaining β-cells in the diabetic animals, most likely through the release of trophic factors since the effects persisted after MSC were no longer detectable. Rackham *et al.*[30] identified annexin A1 (ANXA1) in MSC as an important modulator of glucose-stimulated insulin release from islets *in vitro* and *in vivo*. A recent study from the same group analyzed the expression of mRNA for ligands expressed by adipose-derived human MSC that could interact with G-protein coupled receptors highly expressed by human β-cells.[31] Thirty-six such genes were identified, including ANXA1. In addition to release in soluble form such ligands were also localized within MSC-derived extracellular matrix, and co-culture of MSC matrix with mouse or human islets improved glucose-stimulated insulin secretion. However, the rapid ability of MSC to enhance insulin release is likely to be multifactorial, since MSC-conditioned culture medium has also been shown to contain trophic molecules such as interleukin (IL)-6, IL-8, vascular endothelial growth factor (VEGF)-A, and HGF.[32] Co-culture with islets demonstrated an increased expression of anti-apoptotic genes such as Bcl2 and Bcl-xl, and the VEGFR2 and Tie2 receptors within β-cells.[33] When islets isolated from STZ-diabetic mice were incubated with MSC there was also an

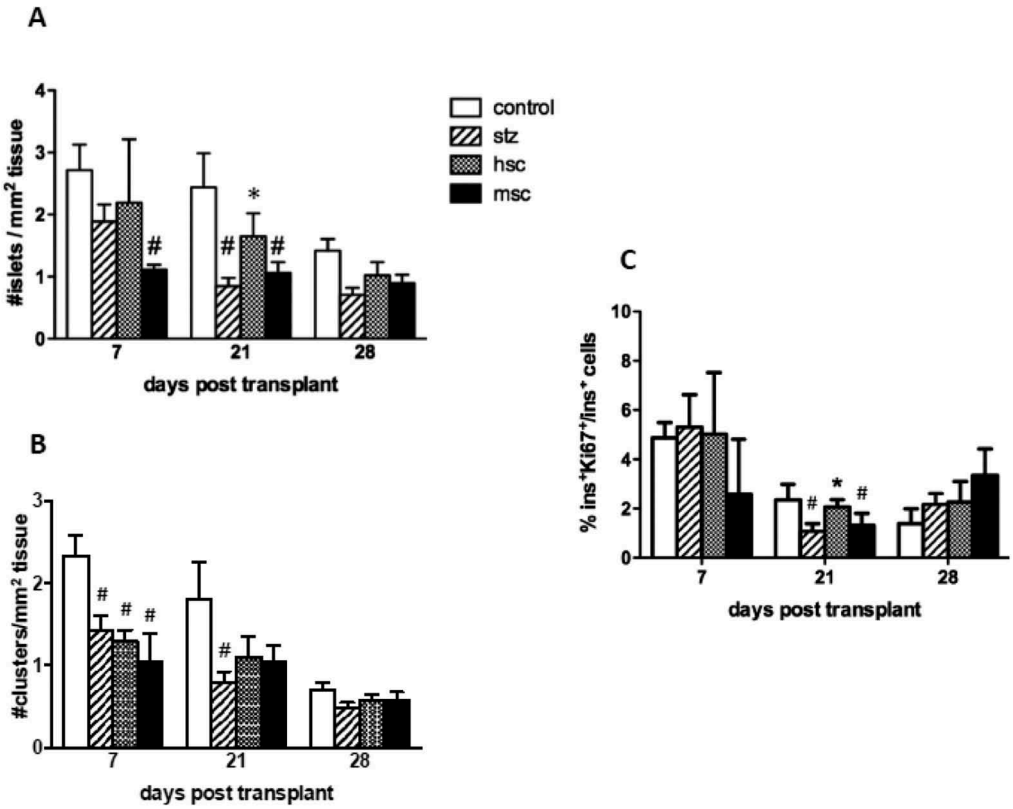

Figure 7. Number of islets (A) or extra-islet endocrine clusters (B) per mm^2 present in pancreata from control mice (citrate buffer injected, sham transplant, open bar), mice treated with STZ (sham transplant, hatched bar), and those who received STZ followed by intra-pancreatic transplant of HSC (stippled bar) or MSC (closed bar) at 7, 21 or 28 days post-grafting. The percent labelling of insulin immunoreactive cells with Ki67 is shown for all ß-cells (C). Values show mean ± SEM; # $p < 0.05$ *vs*. control, * $p < 0.05$ *vs*. STZ alone; n = 4–10 animals per time point.

increased expression of Pdx1, which is necessary for insulin synthesis.[28] The consensus of these and other findings supports the ability of secreted factors from MSC to mobilize increased insulin secretion *in vivo*, but it is unclear if MSC also release proteins that can induce β-cell regeneration. Analysis of human BMSC for their ability to promote β-cell regeneration when transplanted into STZ-diabetic NOD.SCID mice identified a regenerative sub-fraction of cells that selectively activated Wnt signaling pathways within the β-cells, and contributed to their proliferation.[34] Similarly, Park *et al.*[35] demonstrated that co-transplantation of human MSC with isolated islets enhanced islet angiogenesis, which could support their survival and proliferation, and this has been linked by others to signaling *via* the Akt and ERK1/2 pathways in β-cells.[36] It is also possible that MSC induce β-cell neogenesis from the pancreatic ducts following pancreas injury,[37] although this process would be expected to take several weeks before a substantial change in β-cell mass was detected. Additionally, human pancreatic exocrine tissue contains resident MSC that are able to generate endocrine progenitor cell markers following repeated cell passaging *in vitro*.[38] In contrast to our findings a single injection of human MSC caused an increase in β-cell mass in STZ-diabetic mice, due to a suppression of apoptosis.[33] In our experimental design transplantation of MSC occurred 15 days after the final STZ injection, by which time all damaged β-cells would have largely been lost through apoptosis. These experiments would not, therefore, reveal beneficial effects of MSC on β-cell survival. It is possible that MSC derived from different tissue sources might have varying effects or potency on enhancing β-cell mass. Our results show that this did not occur with allografts of bone-marrow-derived MSC in immune-deficient mice over a four week period.

In the present study the improvement of glycemic control was not sustained beyond 21 days

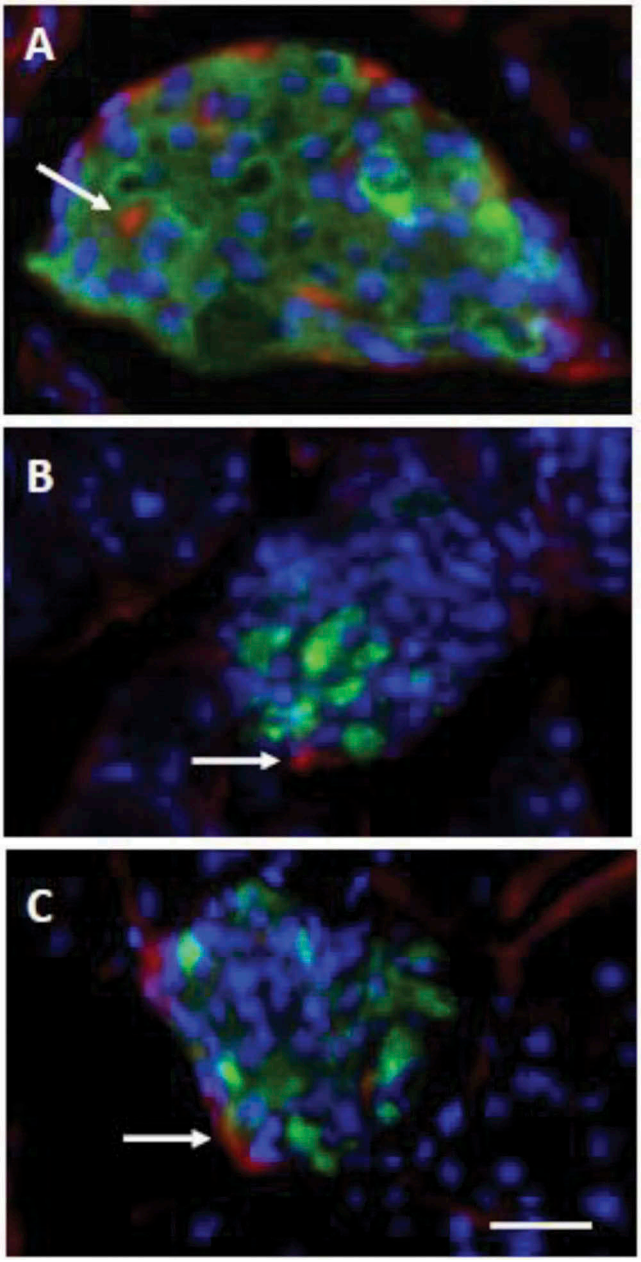

Figure 8. Immunohistochemical localization of CD31 (red, arrows) and insulin (green) within representative islets of Langerhans within the pancreas of control mice (A), mice treated with STZ alone (B), or those given STZ with a subsequent transplant of MSC (C), 21 days after cell transplant. Cell nuclei are visualized using DAPI stain (blue). The magnification bar indicates 100 μm.

following a single treatment with MSC. However, repeated administration of MSC to diabetic immune-competent rats did cause a sustained improvement in both insulin secretion and glucose tolerance.[39] Similarly, in immune-competent diabetic mice administration of MSC resulted in a long-term reversal of hyperglycemia associated

Table 2. Changes in the number of CD31-immunopositive endothelial cells per mm^2 of islet following 21 days following transplant of HSC or MSC into STZ-diabetic NOD.SCID mice compared to non-diabetic control animals.

Treatment	CD31-positive cells
Control	1.11 ± 0.02
STZ	0.07 ± 0.01
MSC	0.06 ± 0.01
HSC	0.06 ± 0.01

Values show ± SEM, n = 4–9 animals

with a reduction in the abundance of autoreactive T cells and a change in the Th1/Th2 ratio towards an anti-inflammatory cytokine profile.[40] The absence of a cytoprotective immunological response in the NOD.SCID may have limited any possible indirect immune effects on β-cell regeneration.

Transplant of HSC into diabetic NOD.SCID mice also improved glycemic control but this was not seen until 21 days post-transplant and was not accompanied by a significant increase in circulating insulin. However, since insulin was measured at the endpoint of a IPGTT the insulin excursion may have already subsided. Unlike MSC, HSC treatment significantly increased β-cell mass to that of control animals, although this was not sustained following a single transplant. The increased β-cell mass was associated with an increase in β-cell proliferation and the number of islets, suggesting that HSC may have enhanced both β-cell neogenesis from progenitors and an increased proliferation in remaining β-cells within islets. This was not associated with an angiogenic effect of HSC since the density of islet endothelial cells was not significantly changed 21 days after transplantation.

Others have also attempted to analyze HSC fractions that promote β-cell regeneration. Bell *et al.*[26] separated human umbilical cord blood stem cells into aldehyde dehydrogenase (ALDH) high or low expressing fractions and tested each for their ability to reverse diabetes in NOD.SCID mice. The ALDH high, but not low cells, demonstrated increased β-cell proliferation and islet vascularization but did not transdifferentiate into insulin-expressing cells. Further characterization showed that the ALDH high cells were enriched for endothelial precursor cells. A subsequent study further identified the active cell fraction as bearing hematopoietic lineage markers

such as CD34 and CD133 but not CD38.[41] A different approach was adopted by Luo *et al.*[42] who separated mouse BMSC using a variety of HSC cell surface markers. A $Sca^{+}/Mac\text{-}1^{-}$ cell fraction was shown to be most effective in infiltrating islets *in vivo*. Finally, small embryonic-like bone marrow-derived HSC with a Fr25 lin^{-} phenotype were shown to be most effective in infiltrating islets and aligning beside potential sites of β-cell neogenesis on pancreatic ducts in the diabetic mouse.[43] The precise signaling mechanisms responsible for initiation of β-cell proliferation by HSC are unknown, but there are a variety of potential target cells. In addition to a direct action on remaining but proliferatively quiescent β-cells, highly proliferative β-cell progenitors have been identified in both the rodent and human pancreas. We identified multi-lineage potential progenitors within islets and in the small, extra-islet endocrine clusters[44] that contain some insulin but poorly express the glucose transporter 2 ($Ins^{+}Glut2^{LO}$ cells), rendering them glucose unresponsive.[45] Under metabolic stress $Ins^{+}Glut2^{LO}$ cells differentiated into mature, functional β-cells. $Ins^{+}Glut2^{LO}$ cells were present in human and mouse pancreas throughout life but decreased in abundance with age.[46] Whilst $Ins^{+}Glut2^{LO}$ cells may represent a resident population of β-cell progenitors there is evidence that at least some may represent a transient phenotype between α-and β-cells. By lineage tagging α-cells in mice a transdifferentiation of α-cells to $Ins^{+}Glut2^{LO}$ cells was demonstrated with the latter being located in clusters and the islet periphery.[47] The α-cell population was subsequently repleted by neogenesis. A similar transdifferentiation was identified in human islets following transplant into immune-deficient animals. Recently, highly proliferative α-cell-like progenitors have also been described in the human pancreas throughout life,[48] as well as newly-committed endocrine progenitors in the mouse pancreas that selectively express high ALDH activity.[49] Either of these populations could be progenitors to $Ins^{+}Glut2^{LO}$ cells, or distinct progenitor pools that are amenable to HSC-induced expansion and differentiation to become functional β-cells.

In summary, we showed that a single intra-pancreatic injection of either MSC or HSC from mouse bone marrow can transiently improve glycemic control in diabetic mouse recipients, but with different time courses and likely different mechanisms of action. We speculate that any contribution of endogenous bone marrow stem cells to the ability of pancreatic β-cells to regenerate, perhaps in early life, will involve the additive action of MSC and HSC.

Compliance with ethical standards

Ethical approval: All animal procedures were approved by the Animal Care Committee of the Western University, Ontario, Canada in accordance with the guidelines of the Canadian Council for Animal Care. This article does not contain any studies with human participants performed by any of the authors.

Abbreviations

7-AAD	7-aminoactinomycin
ALDH	aldehyde dehydrogenase
AUC	area under the curve
BMSC	bone marrow stem cells
FACS	fluorescence-activated cell sorting
FISH	fluorescence in situ hybridization
GFP	green fluorescent protein
HGF	hepatocyte growth factor
HSC	hematopoietic stem cells
IPGTT	intraperitoneal glucose tolerance test
MSC	mesenchymal stem cells
Ngn3	neurogenin3
NOD	non-obese diabetic
Pdx1	pancreatic duodenal homeodomain box 1
SCID	severe combined immunodeficient
SDF-1	stromal cell-derived factor 1
STZ	streptozotocin
VEGF	vascular endothelial growth factor
YFP	Yellow fluorescent protein

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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