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ARTICLE

# Impact of whole body irradiation and vascular endothelial growth factor-A on increased beta cell mass after bone marrow transplantation in a mouse model of diabetes induced by streptozotocin

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**Abstract**
*Aims/hypothesis* Recent studies have shown that bone marrow transplantation reduces hyperglycaemia in a mouse model of diabetes induced by streptozotocin. However, the essential factors for the improvement of hyperglycaemia by bone marrow transplantation have not been fully elucidated. The aim of this study was to search for such factors.

*Methods* We investigated the effect of irradiation to whole body, to abdomen alone or to whole body excluding abdomen, followed by infusion or no infusion of bone marrow cells. We also investigated the effect of bone marrow transplantation on beta cell-specific vascular endothelial growth factor-A gene (*Vegfa*) knockout mice.
*Results* Bone marrow transplantation improved streptozotocin-induced hyperglycaemia and partially restored islet mass. This change was associated with increased islet vascularisation. Among the other methods investigated, low-dose irradiation of the whole body without infusion of bone marrow cells also improved blood glucose level. In streptozotocin-treated beta cell-specific *Vegfa* knockout mice, which exhibit impaired islet vascularisation, bone marrow transplantation neither improved hyperglycaemia, relative beta cell mass nor islet vascularisation.
*Conclusion/interpretation* Our results indicate that whole body irradiation is essential and sufficient for restoration of beta cell mass after streptozotocin treatment independent of infusion of bone marrow cells. Vascular endothelial growth factor-A produced in beta cells is also essential for this phenomenon.

**Keywords** Endothelial progenitor cell · Insulin · Regeneration · Stem cell · VEGF

**Abbreviations**

| | |
|---|---|
| BM | bone marrow |
| BMT | bone marrow transplantation |
| GFP | green fluorescent protein |
| IPGTT | intraperitoneal glucose tolerance test |
| MMP-9 | matrix metalloproteinase-9 |
| sKitL | soluble kit-ligand |
| STZ | streptozotocin |
| VEGF | vascular endothelial growth factor |

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## Introduction

A number of recent studies indicate that bone marrow (BM)-derived cells may give rise to various cell types by a process termed transdifferentiation [1–8]. However, these data are controversial because several other studies report that these cells fail to show such transdifferentiation [9–14]. With regard to pancreatic beta cells, a previous study showed that BM-derived cells can directly transdifferentiate into beta cells [15]. On the other hand, other groups including ours showed that very few or no BM-derived cells can transdifferentiate into insulin-positive cells in islets [16–18]. While the difference between these studies' findings might result from the different procedures used for tracing transplanted BM-derived cells, Taneera et al. [19] systematically compared various approaches that promote transdifferentiation from BM cells to beta cells and demonstrated that BM-derived cells engraft efficiently in the pancreas but adopt almost exclusively a haematopoietic cell fate.

Apart from the issue of cell fate of BM-derived cells, recent studies have suggested that BM transplantation (BMT) promotes endogenous pancreatic beta cell regeneration and results in improvement of hyperglycaemia in a mouse model of diabetes induced by streptozotocin (STZ) [20, 21]. In addition, other studies demonstrated that BMT improves hyperglycaemia in other animal models of diabetes, e.g. *E2f1*/*E2f2* mutant mice [22], and KKAy mice [23]. These data suggest that the improvement of hyperglycaemia by BMT may not be limited to mice with STZ-induced diabetes. On the other hand, a few studies showed that BMT did not improve hyperglycaemia in a mouse model of diabetes [17, 18]. Therefore, it is important to elucidate the reproducibility of the effect of BMT on the reduction of the blood glucose level in diabetic model mice. In addition, the factors involved in the improvement of blood glucose level by BMT have not been elucidated.

In this study, we investigated whether BMT improves the blood glucose level in a mouse model of diabetes and searched for the factors responsible for the improvement of blood glucose level after BMT. To confirm the results of previous studies, we used a mouse model of diabetes induced by STZ in the present study, because this model is the most frequently used in studies of BMT.

## Methods

*Animals* The study protocol was reviewed and approved by the Animal Care and Use Committee of Juntendo University, School of Medicine and Osaka Medical Center for Cancer and Cardiovascular Disease. All mice were housed in specific pathogen-free barrier facilities, with a 12 h light–dark cycle, and free access to standard rodent food (Oriental Yeast, Osaka, Japan) and water. Male C57BL/6J mice were purchased from Japan SLC (Hamamatsu, Japan) at 8 weeks of age. Green fluorescent protein (GFP) transgenic mice, in which enhanced GFP production was under the control of the cytomegalovirus enhancer and the chicken β-actin promoter, were generously provided by M. Okabe (Osaka University, Osaka, Japan) [24]. The generation and characterisation of RIP-CRE transgenic mice [25] and vascular endothelial growth factor ($VEGF^{fl/fl}$) mice ($VEGF^{fl/fl}$ [$CRE^-$] mice) [26] has been reported previously [27, 28]. To generate the mouse model of diabetes, STZ was solubilised in sodium citrate buffer (pH 4.5) and 50 or 100 mg/kg body weight of STZ was injected intraperitoneally into each mouse at the age of 8 weeks daily for 5 days.

*BMT* The standard procedure of BMT as described previously [16] was carried out with 8-week-old GFP transgenic mice or C57BL/6J mice as the donors and 8-week-old C57BL/6J mice, $VEGF^{fl/fl}$ [$CRE^-$] mice or RIP-CRE: $VEGF^{fl/fl}$ mice as the recipients. Some C57BL/6J mice were irradiated (5 or 10 Gy) but this was not followed by infusion of BM cells. In some C57BL/6J mice, either the abdomen only or the whole body except the abdomen was irradiated by masking the region where the irradiation was to be avoided with a radiodense 2 cm-thick lead sheet followed by infusion or no infusion of BM cells as described previously [29, 30].

*Measurement of blood glucose level and serum insulin* We measured blood glucose and serum insulin as described previously [28]. For details on methods, see Electronic supplementary material (ESM).

*Immunohistochemical analysis* Eight weeks after BMT or irradiation the mice were anaesthetised; the pancreas was removed after heart perfusion, and fixed in a solution of 4% (wt/vol.) paraformaldehyde or zinc formalin at 4°C overnight. The fixed tissue was embedded in paraffin and then cut into 5 μm-thick sections, which were mounted on slides. Immunohistochemical analysis was carried out using guinea pig anti-human insulin antibody (Linco Research, St Charles, MO, USA; dilution 1:200), rat anti-mouse CD31 antibody (Pharmingen, San Diego, CA, USA; dilution 1:200), rat anti-mouse CD45 antibody (Pharmingen; dilution 1:500) and mouse anti-human Ki-67 antibody (Pharmingen; dilution 1:1,000) as described previously [28]. The percentage of the area immunopositive for CD31 and the islet beta cell area were determined on CD31- and insulin-stained sections using a microscope (E800; Nikon, Tokyo, Japan). Each area was determined on ten immunostained sections from each mouse, with each section separated by at least 200 μm. The areas of positive staining were automatically measured using image analysis software

(Image Pro 4.5J; Planetron, Tokyo, Japan). Non-specific staining was excluded from the quantification. The percentage areas immunopositive for CD31 represented the immunopositive area relative to the whole islet area. The percentage of beta cell area was calculated using the following formula: islet beta cell area (%)=the area stained by insulin antibody/the whole pancreatic area. For double staining of CD31 with GFP, fluorescent staining was performed as described previously [16]. To evaluate beta cell replication, the number of Ki-67-positive cells per islet was counted using five immunostained sections from each mouse, with each section separated by at least 200 μm. Fluorescent staining of these samples was observed using a Zeiss Axioskop 2 plus microscope (Carl Zeiss, Jena, Germany), and the digital images were captured using Axiovision 3.0 software.

*Data analysis* Results are presented as the mean±SEM. Differences between two groups were examined for statistical significance using the unpaired Student's *t* test. When comparisons of more than three groups were required, statistical significance was determined by one-way ANOVA and Scheffé's method as the post hoc test. A $p$ value <0.05 was considered significant.

## Results

*BMT improves hyperglycaemia in mice with STZ-induced diabetes* In the present study, 8-week-old C57BL/6J mice were injected with STZ daily for 5 days, followed by irradiation (10 Gy) and subsequent infusion of BM cells obtained from GFP transgenic mice (STZ+BMT mice) (Fig. 1a). Whereas STZ-treated mice that underwent neither irradiation nor infusion of BM cells (STZ–BMT mice) showed markedly high non-fasting blood glucose levels, the non-fasting blood glucose levels of STZ+BMT mice were significantly lower than STZ–BMT mice. This effect continued for at least 8 weeks after BMT (Fig. 1b). The intraperitoneal glucose tolerance test (IPGTT) performed 8 weeks after BMT revealed that STZ+BMT mice had improved glucose tolerance with the increase in insulin level during the test (Fig. 1c,d). In contrast, there were no differences in insulin sensitivity judged by insulin tolerance test between STZ+BMT mice and STZ–BMT mice (data not shown). This confirmed that BMT improves hyperglycaemia in mice with STZ-induced diabetes. This improvement was at least in part the result of an improvement in beta cell function.

*BMT increases beta cell mass with increased vascularisation in islets* Next, we analysed pancreatic islets 8 weeks after BMT by immunohistological examination. Staining of

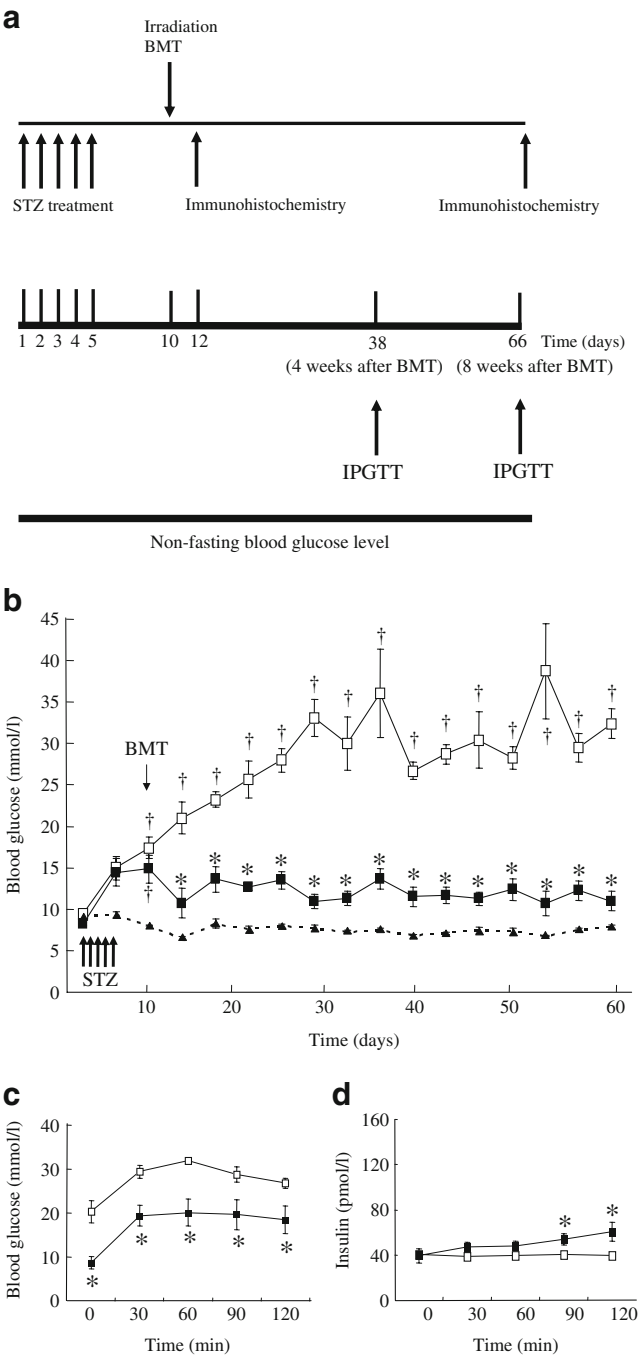


**Fig. 1** Non-fasting blood glucose level and glucose tolerance in STZ-treated C57BL/6J mice with and without BMT. **a** Experimental design for the induction of hyperglycaemia by STZ and BMT and analysis of the effect of BMT. **b** Non-fasting blood glucose in untreated C57BL/6J mice (triangle, $n$=4), STZ-treated C57BL/6J mice with BMT (black squares, $n$=8) and without BMT (white squares, $n$=10). **c**, **d** Results of IPGTT at 8 weeks after BMT in STZ-treated C57BL/6J mice with (black squares, $n$=4) and without (white squares, $n$=4) BMT. Blood glucose levels (**c**) and serum insulin levels (**d**) during IPGTT in each group are shown. Values are mean±SEM. †$p$<0.05 vs non-treated C57BL/6J mice, *$p$<0.05 vs STZ-treated C57BL/6J mice without BMT

the pancreas using anti-insulin antibody showed a larger relative beta cell mass in STZ+BMT mice than in STZ–BMT mice (Fig. 2a,b). Next, we compared relative beta cell mass at 2 days and 8 weeks after BMT in STZ+BMT and STZ–BMT mice. In STZ–BMT mice, there was a significant decrease of relative beta cell mass 8 weeks after BMT compared with that 2 days after BMT. In contrast, in STZ+BMT mice, there was a significant increase of relative beta cell mass 8 weeks after BMT compared with that 2 days after BMT (Fig. 2c). In addition, higher numbers of Ki-67-positive cells were noted in STZ+BMT mice compared with STZ–BMT mice 8 weeks after BMT (Fig. 2d). These results suggest that BMT may augment the relative beta cell mass, rather than simply preventing the destruction of beta cells. Staining of the pancreas using anti-CD31 antibody revealed more endothelial cells in islets of STZ+BMT mice than STZ–BMT mice (Fig. 2e,f). In the next step, we investigated whether the increased endothelial cells in STZ+BMT mice were derived from BM cells. GFP and CD31 double-positive cells were occasionally observed in islets. The frequency of GFP and CD31 double-positive cells in islets was one cell per four to five islets (data not shown), which suggests that BMT increases relative beta cell mass with increased vascularisation in islets, however, BM cells do not seem to provide the bulk of accumulated endothelial cells in islets.

*Irradiation of whole body is essential and sufficient for increased beta cell mass in mice with STZ-induced diabetes* To investigate the process that is most essential and sufficient for the reduction of hyperglycaemia among the BMT protocols, we divided C57BL/6J mice into five groups. In group 1 (control group), 8-week-old mice were treated with STZ daily for 5 days, followed by no procedures. This group is basically the same as the STZ–BMT mice. In group 2, mice were treated with STZ, followed by infusion of BM cells without irradiation. In group 3, mice were treated with STZ, followed by irradiation limited to the abdomen without infusion of BM cells. In group 4, mice were treated with STZ, followed by irradiation limited to the abdomen with infusion of BM cells. In group 5, mice were treated with STZ, followed by irradiation of the whole body except the abdomen with infusion of BM cells (ESM Fig. 1a). There was also a sixth group of mice exposed to whole body lethal irradiation without infusion of BM cells but all of these mice were

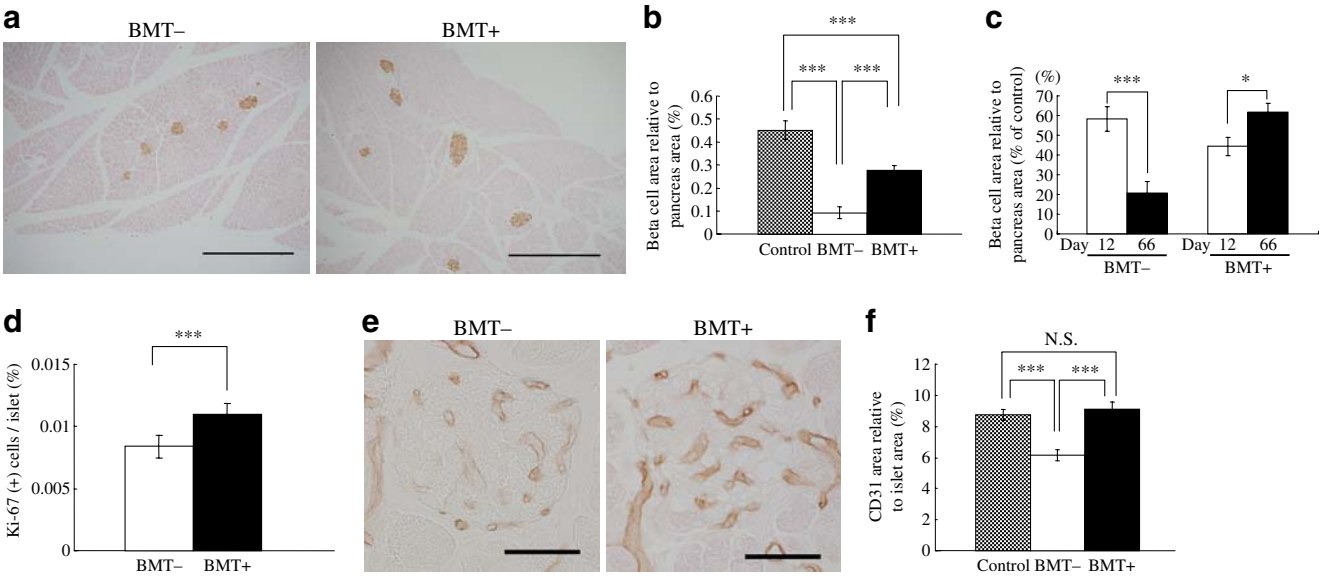


**Fig. 2** Areas of beta cells and endothelial cells in islets of STZ-treated C57BL/6J mice with and without BMT. **a**–**c** Pancreases from non-treated C57BL/6J mice, and STZ-treated C57BL/6J mice with and without BMT were immunostained with an anti-insulin antibody. **a** Typical image of immunohistochemical staining with insulin 8 weeks after BMT in each group. **b** Beta cell area in pancreas of non-treated C57BL/6J mice (grey bar, $n=5$), STZ-treated C57BL/6J mice with (black bar, $n=6$) and without (white bar, $n=7$) BMT at 8 weeks after BMT. **c** Beta cell area in pancreas of STZ-treated C57BL/6J mice with BMT at 2 days (white bar, $n=4$) and 8 weeks (black bar, $n=6$) after BMT and the corresponding time-point of STZ-treated C57BL/6J mice without BMT (12 days after STZ treatment, white bar, $n=4$; 66 days after STZ treatment, black bar, $n=7$). Each beta cell area was represented as a relative value to non-treated mice. **d** Pancreas of STZ-treated C57BL/6J mice with and without BMT were immunostained for Ki-67 antibody. Number of Ki-67-positive intra-islet cells in pancreas of STZ-treated C57BL/6J mice with (black bar, $n=5$) and without (white bar, $n=5$) BMT at 8 weeks after BMT. **e**, **f** Pancreas of non-treated C57BL/6J mice and of STZ-treated C57BL/6J mice with and without BMT were immunostained for CD31. **e** Typical image of immunohistochemical staining with CD31 8 weeks after BMT in each group. **f** Percentages of CD31-immunopositive areas in islets of non-treated C57BL/6J mice (grey bar, $n=4$), STZ-treated C57BL/6J mice with (black bar, $n=4$) and without (white bar, $n=4$) BMT at 8 weeks after BMT. Values are mean±SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$. Scale bars: **a** 500 μm; **e** 50 μm. N.S., not significant

dead within 1 week after irradiation. As shown in ESM Fig. 1b, no significant reduction in blood glucose level was observed in any of the groups, compared with group 1, suggesting that irradiation of the whole body is essential for reduction of hyperglycaemia by BMT.

Next, to investigate whether whole body irradiation is sufficient for the improvement of hyperglycaemia by BMT, we exposed a group of mice to low-dose irradiation (5 Gy) without infusion of BM cells and compared the blood glucose lowering effect with that in the control mice. As shown in Fig. 3, blood glucose level was lower in mice exposed to low-dose irradiation than in control mice. Improved glucose tolerance with a modest increase in insulin secretion was observed at 8 weeks after irradiation (ESM Fig. 2a). Whereas low-dose irradiation used alone was less effective in improving glucose tolerance than lethal-dose irradiation followed by infusion of BM cells, it definitely improved glucose tolerance. Immunohistological analysis of pancreatic islets demonstrated that low-dose irradiation significantly increased relative beta cell mass (ESM Fig. 2b). This change was associated with a significant increase in islet endothelial cells, similar to the change seen in islets after BMT (ESM Fig. 2c). These results indicate that irradiation of the whole body is essential and sufficient to the relative increase of beta cell mass in STZ-treated mice; whereas infusion of BM cells either with or without reconstitution of the bone marrow is not essential for improvement in blood glucose level.

*VEGF-A in beta cells is essential for the blood glucose lowering effect of BMT* After both BMT and low-dose irradiation, the reduction in blood glucose level was associated with a relative increase of beta cell mass and

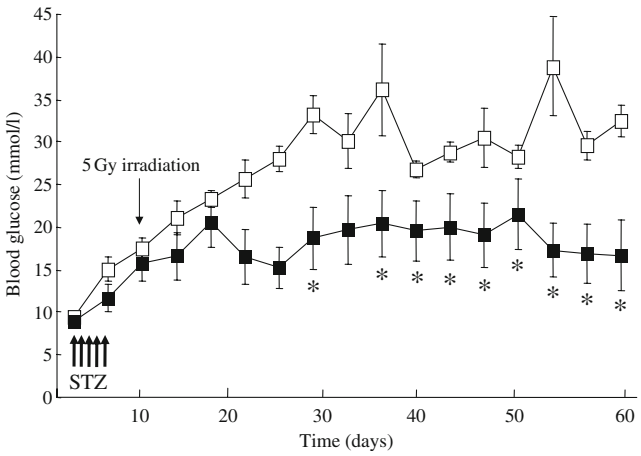


**Fig. 3** Effects of low-dose irradiation on blood glucose level. Comparison of non-fasting blood glucose in STZ-treated C57BL/6J mice with 5 Gy irradiation (black squares, $n=13$) and without irradiation (white squares, $n=10$). Values are mean±SEM. *$p<0.05$ vs STZ-treated C57BL/6J mice without irradiation

vascularisation in islets. Previously we found that VEGF-A produced by beta cells was an essential factor for islet vascularisation [27, 28]. To investigate the role of VEGF-A in beta cells, we used RIP-CRE:VEGF$^{fl/fl}$ mice in which the gene encoding VEGF-A (*Vegfa*) is disrupted in the beta cells and their control mice, VEGF$^{fl/fl}$ [CRE$^-$] mice [27]. As we showed previously [28], a reduced number of endothelial cells and abnormal residual endothelial cells were noted in RIP-CRE:VEGF$^{fl/fl}$ mice. Both the RIP-CRE:VEGF$^{fl/fl}$ mice and the VEGF$^{fl/fl}$ [CRE$^-$] mice (8 weeks old) were injected with 50 mg/kg STZ daily for 5 days; this was followed by irradiation with BMT or no irradiation without BMT. We then evaluated the blood glucose lowering effect of BMT in each mouse. Similar to standard C57BL/6J mice, BMT reduced non-fasting blood glucose levels in control VEGF$^{fl/fl}$ [CRE$^-$] mice (Fig. 4a). On the other hand, in RIP-CRE:VEGF$^{fl/fl}$ mice, no reduction in non-fasting blood glucose level by BMT was observed under the same experimental procedures (Fig. 4b). One of the differences between RIP-CRE:VEGF$^{fl/fl}$ mice and their control VEGF$^{fl/fl}$ [CRE$^-$] mice was the effect of STZ on blood glucose level. Although the same dose of STZ was used, STZ was less effective in increasing blood glucose in RIP-CRE:VEGF$^{fl/fl}$ mice. It is likely that in RIP-CRE:VEGF$^{fl/fl}$ mice, STZ may not reach the islets efficiently because of low blood flow in the islets. To investigate the possibility that the difference in blood glucose lowering effect of BMT between RIP-CRE:VEGF$^{fl/fl}$ mice and their control VEGF$^{fl/fl}$ [CRE$^-$] mice was the result of the effect of STZ on blood glucose level, we increased the dose of STZ and re-evaluated the effect of BMT on RIP-CRE:VEGF$^{fl/fl}$ mice. The blood glucose level in RIP-CRE:VEGF$^{fl/fl}$ mice injected with 100 mg/kg STZ for 5 days was similar to that of VEGF$^{fl/fl}$ [CRE$^-$] mice injected with 50 mg/kg STZ for 5 days. Under these conditions, BMT also failed to reduce blood glucose level in RIP-CRE:VEGF$^{fl/fl}$ mice (Fig. 4c). These results confirmed the lack of reduction of non-fasting blood glucose level by BMT in RIP-CRE:VEGF$^{fl/fl}$ mice. The IPGTT at 4 and 8 weeks after BMT showed that BMT improved glucose tolerance in VEGF$^{fl/fl}$ [CRE$^-$] mice but not in RIP-CRE:VEGF$^{fl/fl}$ mice (Fig. 5, and data not shown).

*VEGF in beta cells is essential for the increase in beta cell mass by BMT* Next, we analysed pancreatic islets of RIP-CRE:VEGF$^{fl/fl}$ mice and their control VEGF$^{fl/fl}$ [CRE$^-$] mice by immunohistological examination. In VEGF$^{fl/fl}$ [CRE$^-$] mice, 50 mg/kg STZ treatment reduced, whereas STZ+BMT restored, endothelial cells in islets. The endothelial cell mass in islets was smaller in RIP-CRE:VEGF$^{fl/fl}$ mice (3.4%) than VEGF$^{fl/fl}$ [CRE$^-$] mice (10.0%; $p<0.05$%). In RIP-CRE:VEGF$^{fl/fl}$ mice, neither 50 mg/kg STZ treatment nor BMT after STZ treatment altered the endothelial cell mass (Fig. 6).

With regard to relative beta cell mass, 50 mg/kg STZ significantly decreased the beta cell mass, while BMT partially restored the decreased beta cell mass in VEGF$^{fl/fl}$ [CRE$^{-}$] mice. On the other hand, in RIP-CRE:VEGF$^{fl/fl}$ mice, both dosages of STZ significantly decreased beta cell mass, but BMT did not restore the decreased beta cell mass (Fig. 7). Therefore the BMT did not result in an increase in vascularisation and relative beta cell mass in STZ-treated RIP-CRE:VEGF$^{fl/fl}$ mice.

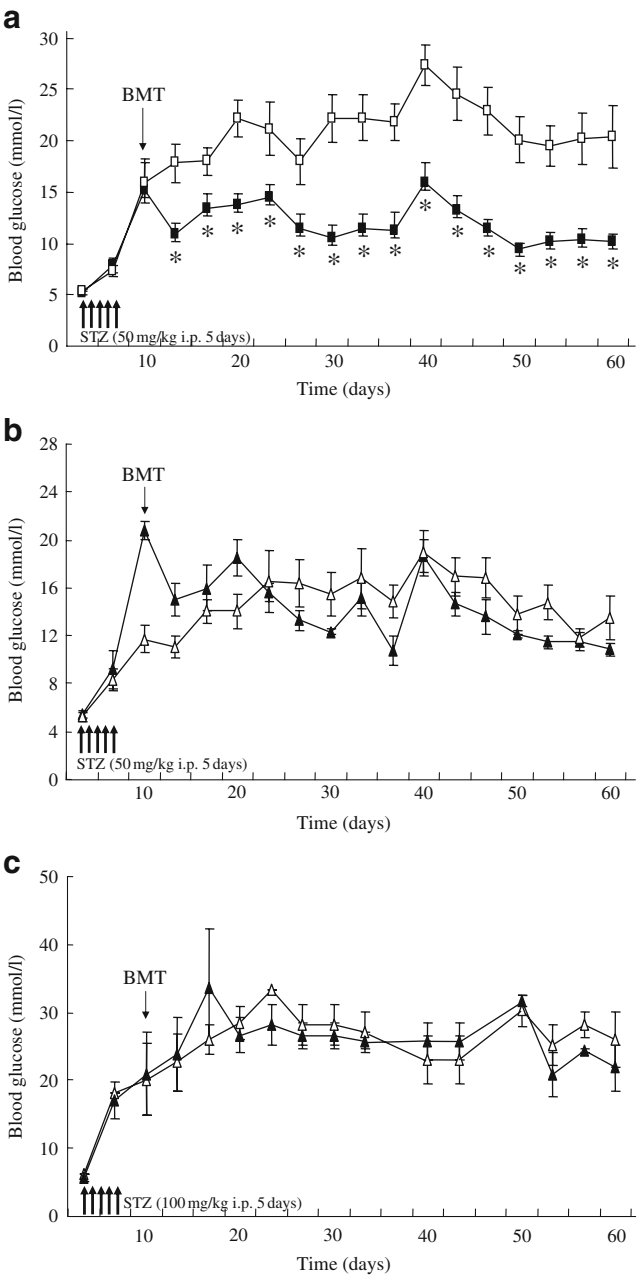


**Fig. 4** Effects of BMT on blood glucose in VEGF$^{fl/fl}$ (Cre$^{-}$) and RIP-Cre:VEGF$^{fl/fl}$ mice. **a** Non-fasting blood glucose levels in STZ-treated VEGF$^{fl/fl}$ (Cre$^{-}$) mice with BMT (black squares, $n$=7) and without BMT (white squares, $n$=6). **b** Non-fasting blood glucose levels in 50 mg/kg STZ-treated RIP-Cre:VEGF$^{fl/fl}$ mice with BMT (black triangles, $n$=7) and without BMT (white triangles, $n$=6). **c** Non-fasting blood glucose levels in 100 mg/kg STZ-treated RIP-CRE:VEGF$^{fl/fl}$ mice with BMT (black triangles, $n$=4) and without BMT (white triangles, $n$=6). Values are mean±SEM. *$p$<0.05 vs STZ-treated corresponding mice without BMT

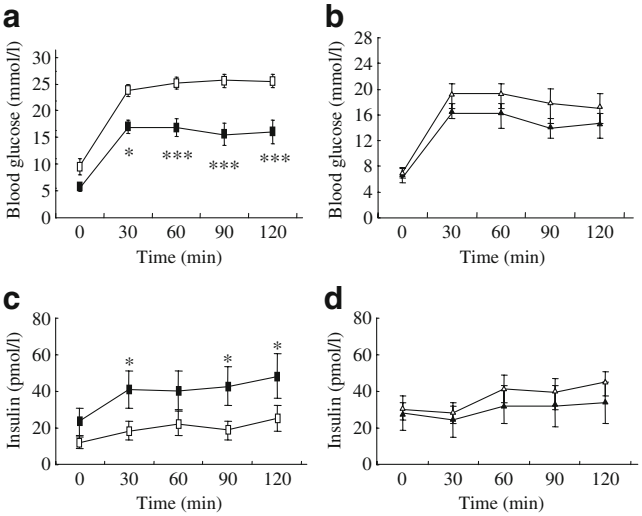


**Fig. 5** Effects of BMT on glucose tolerance in VEGF$^{fl/fl}$ (Cre$^{-}$) and RIP-Cre:VEGF$^{fl/fl}$ mice. Blood glucose (**a**, **b**) and serum insulin level (**c**, **d**) during IPGTT at 8 weeks after BMT in STZ-treated VEGF$^{fl/fl}$ (Cre$^{-}$) mice (**a**, **c**) and RIP-CRE:VEGF$^{fl/fl}$ mice (**b**, **d**) with BMT (black squares; VEGF$^{fl/fl}$ (Cre$^{-}$) mice, $n$=7; RIP-CRE:VEGF$^{fl/fl}$ mice, $n$=4) and without BMT (white squares; VEGF$^{fl/fl}$ (Cre$^{-}$) mice, $n$=10; RIP-CRE:VEGF$^{fl/fl}$ mice, $n$=8). Values are mean±SEM. *$p$<0.05, **$p$<0.01, ***$p$<0.001 vs STZ-treated corresponding mice without BMT

## Discussion

In the present study, we demonstrated that BMT has a blood glucose lowering effect in mice with STZ-induced diabetes. This effect is associated with increased relative beta cell and endothelial cell mass in islets. We also investigated the essential factors for the BMT-induced increase in relative beta cell mass in mice with STZ-induced diabetes in this study.

Whereas the low-dose STZ model is potentially a model of immune-mediated beta cell destruction [31], we found the significant increase of relative beta cell mass with the increase of the cell proliferation rate at 8 weeks after BMT, compared with 2 days after BMT (Fig. 2c,d). This result suggests that the restoration of relative beta cell mass by BMT could be more the result of the augmentation of relative beta cell mass than of the simple prevention of immune-mediated destruction of beta cells. This finding is also supported by other studies demonstrating that BMT improves hyperglycaemia in other types of diabetic animals: e.g. *E2f1*/*E2f2* mutant mice [22] and KKAy mice [23].

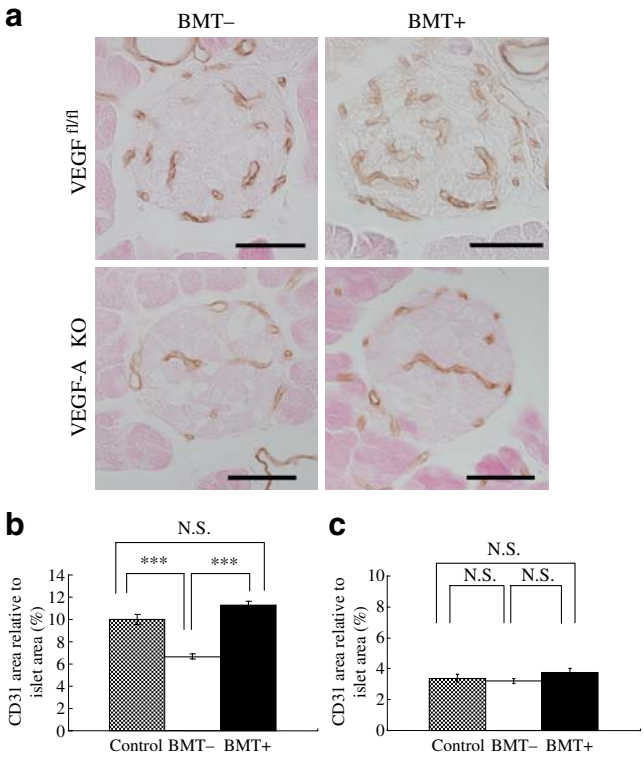


**Fig. 6** Effects of BMT on islet vascularisation in VEGF$^{fl/fl}$(Cre$^{-}$) and RIP-CRE:VEGF$^{fl/fl}$ mice. **a** Micrographs of pancreas of representative 50 mg/kg STZ-treated VEGF$^{fl/fl}$ (Cre$^{-}$) and RIP-CRE:VEGF$^{fl/fl}$ mice with and without BMT immunostained for CD31. Scale bars: 50 μm. **b** Percentages of CD31-immunopositive areas in islets of VEGF$^{fl/fl}$ (Cre$^{-}$) mice (grey bar, $n$=5), 50 mg/kg STZ-treated VEGF$^{fl/fl}$ (Cre$^{-}$) mice without BMT (white bar, $n$=6) and 50 mg/kg STZ-treated VEGF$^{fl/fl}$ (Cre$^{-}$) mice with BMT (black bar, $n$=6). **c** Percentages of CD31-immunopositive areas in islets of RIP-CRE:VEGF$^{fl/fl}$ mice (grey bar, $n$=5), 50 mg/kg STZ-treated RIP-CRE:VEGF$^{fl/fl}$ mice without BMT (white bar, $n$=6) and 50 mg/kg STZ-treated RIP-CRE:VEGF$^{fl/fl}$ mice with BMT (black bar, $n$=7). Values are mean±SEM. *$p$<0.05, **$p$<0.01, ***$p$<0.001

BMT encompasses the processes of lethal irradiation followed by infusion of BM cells. In this process, the recipient BM cells are replaced by donor BM cells. During this process, after myelosuppression of the recipient, the donor BM cells home to the BM and progenitor cells mobilise and expand in the peripheral blood [32]. Previous studies demonstrated that irradiation of bone marrow cells alone induces mobilisation and expansion of progenitor cells in the peripheral blood [33]. In addition, regional irradiation increases the gene expression of cytokines, growth factors and transcription factors such as TNF-α, IL-1, fibroblast growth factor and nuclear factor-κB [34–37]. Therefore, among the procedures of BMT, it is possible that both irradiation of the BM and of the pancreas may play important roles in the relative increase of beta cell mass. In this study, to elucidate the importance of irradiation to each of these two components, we used mice that had received irradiation of the whole body excluding the abdomen, and irradiation limited to the abdomen. Irradiation of the abdomen only, followed by infusion of BM cells had no lowering effect on blood glucose, clearly indicating that irradiation of the pancreas alone does not result in lowering of blood glucose levels. In contrast, whole body irradiation excluding the abdomen followed by infusion of BM cells also had no blood glucose lowering effect, suggesting that BM irradiation alone is also ineffective in reducing the blood glucose level and that another essential process is the irradiation of the abdomen including the pancreas. We were unable to direct the irradiation to include the whole body but exclude only the pancreas because of technical difficulties so we cannot completely exclude the possibility that irradiation of abdominal organs other than the pancreas is important for the reduction of blood glucose level. However, considering that reduction of blood glucose was achieved by restoration

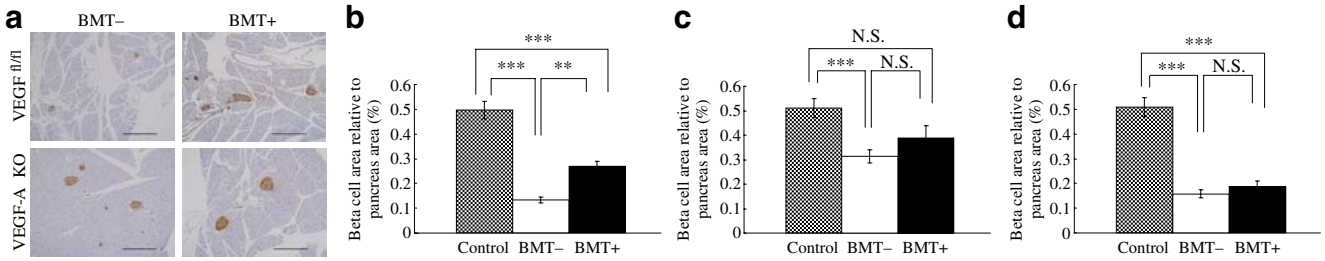


**Fig. 7** Effects of BMT on islet vascularisation and beta cell mass in VEGF$^{fl/fl}$ (Cre$^{-}$) and RIP-CRE:VEGF$^{fl/fl}$ mice. **a** Micrographs of pancreas of representative 50 mg/kg STZ-treated VEGF$^{fl/fl}$ (Cre$^{-}$) and RIP-CRE:VEGF$^{fl/fl}$ mice with and without BMT were immunostained for insulin. Scale bars: 200 μm. **b** Percentages of insulin-immunopositive areas in pancreas of VEGF$^{fl/fl}$ (Cre$^{-}$) mice (grey bar, $n$=8), 50 mg/kg STZ-treated VEGF$^{fl/fl}$ (Cre$^{-}$) mice without BMT (white bar, $n$=6) and 50 mg/kg STZ-treated VEGF$^{fl/fl}$ (Cre$^{-}$) mice with BMT (black bar, $n$=6). **c** Percentages of insulin-immunopositive areas in pancreas of RIP-CRE:VEGF$^{fl/fl}$ mice (grey bar, $n$=9), 50 mg/kg STZ-treated RIP-Cre:VEGF$^{fl/fl}$ mice without BMT (white bar, $n$=7) and STZ-treated RIP-Cre:VEGF$^{fl/fl}$ mice with BMT (black bar, $n$=5). **d** Percentages of insulin-immunopositive areas in pancreas of RIP-CRE:VEGF$^{fl/fl}$ mice (grey bar, $n$=9), 100 mg/kg STZ-treated RIP-CRE:VEGF$^{fl/fl}$ mice without BMT (white bar, $n$=5) and 100 mg/kg STZ-treated RIP-CRE:VEGF$^{fl/fl}$ mice with BMT (black bar, $n$=5). Values are mean±SEM. *$p$<0.05, **$p$<0.01, ***$p$<0.001. N.S., not significant

of beta cell function, it is likely that irradiation of the pancreas is involved in BMT-related reduction in blood glucose levels.

To investigate whether irradiation to the whole body is sufficient to lower the blood glucose level, STZ-treated mice were exposed to lethal (10 Gy) and sublethal (5 Gy) irradiation without subsequent BM cell infusion. All mice exposed by 10 Gy irradiation died within 1 week so we cannot assess the blood glucose lowering effect of this dose of irradiation. However, mice exposed to 5 Gy irradiation had reduced hyperglycaemia and increased relative beta cell mass, clearly demonstrating that irradiation of the whole body is essential and sufficiently explains the blood glucose lowering effect of BMT. The blood glucose lowering effect of 5 Gy irradiation was less efficient than that of 10 Gy irradiation followed by BMT. This might be linked to the low dose of irradiation itself, although we cannot completely exclude the possibility that infusion of BM cells may have additional effect(s) on the whole body irradiation blood glucose lowering effect. Nevertheless, our data clearly demonstrate that infusion of BM cells by itself has no effect on the blood glucose lowering effect of BMT.

Irradiation of the BM induced mobilisation of BM cells. The mechanisms of such mobilisation have been studied in detail. After irradiation, secreted cytokines/chemokines, such as stromal cell-derived factor and VEGF, activate matrix metalloproteinase 9 (MMP-9) in the BM microenvironment [33]. Activated MMP-9 processes membrane-bound kit-ligand, releasing it as soluble kit-ligand (sKitL), which then binds to *c-Kit* (also known as *Kit*) on the surface of stem cells with stimulation of their mobilisation from the BM [38]. Because nitric oxide from BM is necessary for MMP-9 activation, sKitL production and the resultant mobilisation of BM-derived cells are impaired in nitric oxide synthase 3 deficient ($Nos3^{-/-}$) mice [39]. Recently, Hasegawa et al. [21] reported that BMT reduces blood glucose levels in wild-type mice but results in neither reduction of blood glucose level nor increase in beta cell mass in $Nos3^{-/-}$ mice. These results support the notion that BMT-induced BM cell mobilisation is essential for the increase of beta cell mass. Taken together with our data, BM cell mobilisation by bone marrow irradiation seems to be an essential process in the blood glucose lowering effect of BMT.

However, BMT-induced BM cell mobilisation is not sufficient for lowering blood glucose level; irradiation of the pancreas appears to be essential for the blood glucose lowering effect of BMT. Considering that irradiation results in the induction of cytokines and growth factors [34–37], irradiation may invoke the activated signal transduction pathway in the pancreas to reduce blood glucose levels. To identify the signal involved is the next target of our research. It is likely that interaction between mobilised BM-derived cells and irradiation-activated signal pathways in the pancreas plays a key role in the increase of relative beta cell mass by BMT.

In the present study, BMT or low-dose irradiation resulted in an increase in endothelial cell mass in the islets. BM-derived cells contain endothelial cell precursor cells; however, most endothelial cells in islets were not derived from BM cells. This observation is consistent with the findings of previous reports [19, 21], and suggests that irradiation increases the proliferation of endothelial cells in islets probably through interaction between the mobilised BM-derived cells and the pancreas irradiation-related activation of signal pathways.

The microvasculature in islets is important for islet development, insulin gene expression, and beta cell proliferation [40–42]. Nikolova et al. [42] indicated that laminins, vascular basement proteins, which are generated by islet endothelial cells are essential for the proliferation of beta cells through $\beta_1$-integrin. In the present study, the increase in islet mass by BMT or low-dose irradiation was associated with increased islet vascularisation. Previously we showed that production of VEGF-A by beta cells is an essential factor for islet vacularisation [27, 28]. In contrast, low-dose irradiation reportedly promotes VEGF-A release from mast cells that migrate to various tissues [33]. In this study, we investigated the relative beta cell mass in RIP-CRE:$VEGF^{fl/fl}$ mice after BMT. BMT in these mice did not result in a lowering of the blood glucose level so VEGF-A in beta cells plays an essential role in the relative increase in beta cell mass after BMT. It is likely that the increased number of endothelial cells linked to VEGF-A plays an important role in this phenomenon. Alternatively, VEGF-A in islets may play other roles than in the increase endothelial cells in beta cells.

Three days after BMT, there is already a significant decrease of blood glucose level in STZ+BMT mice compared with STZ–BMT mice (Fig. 1b), although the relative beta cell mass in STZ+BMT mice was not significantly different from that in STZ–BMT mice (Fig. 2c) at that time. This result suggests that in addition to the increase of beta cell mass, BMT may have the effect of improvement of beta cell function, independent of the effect on beta cell mass. Elucidation of molecular mechanisms underlying this effect awaits further investigation.

In summary, we confirmed that BMT increased relative beta cell mass in mice with STZ-induced diabetes. This increase seems to be dependent on an increase in endothelial cell mass induced by irradiation of the BM and pancreas. Full elucidation of the mechanism of increased beta cell mass after BMT could eventually contribute to the design of a new strategy to increase beta cell mass, which is one of the main abnormalities in both type 1 and type 2 diabetes.

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