



## Original article

## Oxidative stress tolerance of early stage diabetic endothelial progenitor cell



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## ABSTRACT

**Introduction:** One of the causes for poor vasculogenesis of diabetes mellitus (DM) is known to rise from the dysfunction of bone marrow-derived endothelial progenitor cells (BM EPCs). However, the origin of its cause is less understood. We aimed to investigate the effect of oxidative stress in early stage of diabetic BM-EPC and whether its vasculogenic dysfunction is caused by oxidative stress.

**Methods:** Bone marrow c-Kit+Sca-1+Lin- (BM-KSL) cells were sorted from control and streptozotocin-induced diabetic C57BL/6J mice by flow cytometry. BM-KSLs were then assessed for vasculogenic potential (colony forming assay; EPC-CFA), accumulation of intracellular ROS (CM-H2DCFDA), carbonylated protein (ELISA), anti-oxidative enzymes expression (RT-qPCR) and catalase activity (Amplex Red).

**Results:** Compared to control, DM BM-KSL had significantly lower EPC-CFUs in both definitive EPC-CFU and total EPC-CFU ( $p < 0.05$ ). Interestingly, the oxidative stress level of DM BM-KSL was comparable and was not significantly different to control followed by increased in anti-oxidative enzymes expression and catalase activity.

**Conclusions:** Primitive BM-EPCs showed vasculogenic dysfunction in early diabetes. However the oxidative stress is not denoted as the major initiating factor of its cause. Our results suggest that primitive BM-KSL cell has the ability to compensate oxidative stress levels in early diabetes by increasing the expression of anti-oxidative enzymes.

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## 1. Introduction

Dysfunction of endothelial progenitor cell (EPC) is thought to be the underlying mechanism of neovascularization impairment and

vascular complications in diabetes. EPC was first described as a specialized subset of hematopoietic progenitor cells, reside in bone marrow as a pool, circulate in peripheral blood and facilitate neovascularization in physiological and pathological conditions [1,2].

**Abbreviations:** BM-KSL, bone marrow derived-c-Kit+Sca-1+Lin-; BM-EPC, bone marrow-derived endothelial progenitor cells; DM BM-KSL, diabetic BM-KSL; EPC-CFA, endothelial progenitor cell colony forming assay; EPC-CFU, endothelial progenitor cell colony forming unit; QQC, quality and quantity culture system; pEPC-CFU, primitive/small EPC-CFU; dEPC-CFU, definitive/large EPC-CFU; tEPC-CFU, total EPC-CFU.

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Consistent data have supported that EPC is dysfunctional in diabetes, as represented by impairment in proliferation [3–5], migration/mobilization [6–8], colony forming capacity [7–9], and tubular formation [6].

Oxidative stress is thought as one of the factors responsible for impairment of neovascularization and complications in diabetes [10–12]. Reactive oxygen species (ROS) induced in diabetes down regulates HIF-1 $\alpha$  and weakening of SDF-1 $\alpha$  and VEGF signaling which disturb cells mobilization into injury site [13,14]. ROS also thought to be responsible for EPC reduction in diabetes through ROS-mediated apoptosis [15]. Nevertheless, previous studies on the effect of oxidative stress on EPC dysfunction are performed on differentiated EPC from cultured of peripheral blood (PB) or BM mononuclear cells. The study of oxidative stress on early stage diabetic EPCs from BM hematopoietic population is not yet elucidated.

Recently Masuda et al., developed an EPC-colony forming assay (EPC-CFA) to distinguish vasculogenic function of primitive or early stage EPCs from hematopoietic stem cell population such as c-Kit+/Sca-1+/Lin- (KSL) cells in mice and CD34+ or CD133+ cells in human. EPC colony forming unit (EPC-CFU) consists of two different EPC colonies: small (primitive; pEPC-CFU) colonies consist of highly immature and proliferative population of EPCs and large (definitive; dEPC-CFU) with high differentiation potential in ready state for vasculogenesis [7,16,17].

Using EPC-CFU, our group reported vasculogenic impairment of early stage diabetic BM-KSL (DM BM-KSL) by decreased number of definitive and total EPC-CFU [7,9], however the mechanism or the direct cause is not yet elucidated. Since previous reports show that oxidative stress is one of the causes of vasculogenic impairment in cultured EPCs, herein we will explore the effect of oxidative stress on vasculogenic potential of diabetic BM vascular progenitor cells from KSL cell population. Using QQc system as facilitating tool, we aim to investigate whether improvement in diabetic EPC post-QQc will follow by decrease in oxidative stress level. To our knowledge, this is the first study to investigate whether diabetic vasculogenic dysfunction resides on the oxidative stress damage of primitive bone marrow EPCs.

## 2. Materials and methods

### 2.1. Animals and STZ-induced diabetic mouse model

All the experimental protocols described were approved by the Animal Care Committees of Juntendo University. We used male 8 weeks old C57BL/6J (20–25 g) mice (Sankyo Laboratory) which were housed in specific pathogen-free barrier facilities and maintained under a 12-hour light/dark cycle. Diabetes (DM) were induced by intraperitoneal injection of Streptozotocin (STZ, 50 mg/kg, Sigma) dissolved in citrate buffer (pH 4.5) for 5 consecutive days, as previously described [9,18]. Mice with blood glucose level equal or higher than 300 mg/dl were decided as diabetic mice which maintained at least for 4 weeks.

### 2.2. Bone marrow (BM) derived KSL cells isolation

BM cells were harvested from femur, tibia, pelvis and humerus bones of DM and control mice as previously described [9]. Erythrocytes were removed by ammonium chloride. The cells were then labeled by biotin-conjugated antibody cocktail (CD3e, CD45/B220, Ly-6G and Ly-6C, CD-11b, TER-119) (all antibodies obtained from eBioscience) and followed by anti-biotin micro-beads depletion by AutoMACS (Miltenyi) to obtain lineage negative (Lin-) cells. The Lin- cells were stained with APC-labeled Sca-1 and PE-labeled c-Kit antibodies (eBioscience), and sorted for c-Kit+Sca-1+Lin- (KSL) cells by FACS Aria (Becton Dickinson).

### 2.3. Serum free quantity and quality culture (QQc) system

Previously our group established a serum-free quality and quantity culture (QQc) system containing thrombopoietin (TPO), VEGF, Stem Cell Factor (SCF), IL-6 and Flt-3 ligand as an optimal quality and quantity culture for EPC expansion to enhance their vasculogenic and regenerative potential [19]. BM-KSL of  $1 \times 10^3$  cells were cultured in QQc system as previously described [9,19,20]. Seven days later cells were harvested and the collected cells were grouped accordingly as DM post-QQc for DM BM-KSL cells.

### 2.4. EPC colony formation assay (EPC-CFA)

EPC vasculogenic CFA was performed and characterized as previously described [7,9,16,17,20]. Briefly a total of 500 BM-KSL cells/dish suspends in semi-solid culture medium for EPC-CFA and were seeded into 35 mm primary culture dish. The frequency of primitive/small (pEPC-CFU), definitive/large (dEPC-CFU) and total colony forming unit (tEPC-CFU) were assessed on day-7 by two investigators who were blinded to the experimental conditions. Experiments were performed in triplicate.

### 2.5. Intracellular ROS detection

Intracellular ROS levels were evaluated with 5-(and -6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H<sub>2</sub>DCFDA, Invitrogen) by followed the manufacture's protocol. Freshly prepared BM-KSL and post-QQc BM-KSL cells from control and or diabetic mice were labeled with CM-H<sub>2</sub>DCFDA and incubated for 15 min at 37 °C. The CM-H<sub>2</sub>DCFDA labeled cells were then analyzed using FACS Calibur (Becton Dickinson).

### 2.6. Protein carbonyl detection for oxidative stress marker

Protein was extracted with RIPA buffer (Thermo Scientific) with protease inhibitor cocktail (Roche). Protein was derivatized with DNPH (2,4-dinitrophenyl hydrazine) as previously described [21]. After removing the excess of DNPH, 96-well ELISA plates were coated at 4 °C overnight with samples. After removing them, the wells were blocked with 3%BSA/PBS at 37 °C for 1 h. Anti DNPH antibody (1:150, Millipore) was added and incubated at 37 °C for 1 h. HRP-labeled anti-rabbit antibody (1:300, Millipore) was added and incubate at 37 °C for 1 h (both first and secondary antibodies were part of Oxyblot Kit, Millipore, Chemicon International). To develop color the o-phenyldiamin dihydrochloride (Sigma) was added for 40 min and stopped by 1 N sulfuric acid. The absorbance was measured at 492 nm [22]. The result presents as ratio of carbonylated protein.

### 2.7. Quantitative real-time PCR (RT-qPCR)

Total RNA was extracted using Trizol (Invitrogen) following the manufacturer's protocol. cDNA was synthesized using SuperScript First-Strand Synthesis System (Invitrogen). The genes expression was measured on an ABI 7500 FAST Real-Time PCR system using TaqMan probes 18S rRNA (ribosomal RNA control reagents), MnSOD (Sod2, Mm01313000\_m1), catalase (Cat, Mm004379 92\_m1) and glutathione Peroxidase-1 (Gpx1, Mm00656767\_g1) (Life Technologies). Relative expression of the target gene was demonstrated by the  $\Delta\Delta Ct$ .

### 2.8. Catalase activity

BM-KSL cells (as minimal  $2 \times 10^4$  cells) were extracted in 0.2% Triton X-100 in 0.1 M phosphate buffer (pH 7.7) and extracts were

prepared on a 96-well plate and incubate with 40  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 30 min at room temperature. Catalase activity was measured as the residual  $\text{H}_2\text{O}_2$  levels by Amplex red catalase assay kit (Invitrogen) following the manufacture's protocol.

## 2.9. Microarray analysis

RNA was isolated from sorted fresh BM-KSL from both control and diabetic group, using RNeasy Micro Kit (Qiagen) according to manufacturer's protocols. Microarray was performed using one color microarray-based gene expression analysis (Agilent Technologies) as described in manufacturer's instructions. The expression values for the genes were determined using Gene Spring GX software version 11. The genes analysis was based on ontology analysis. All experiments were done in duplicates.

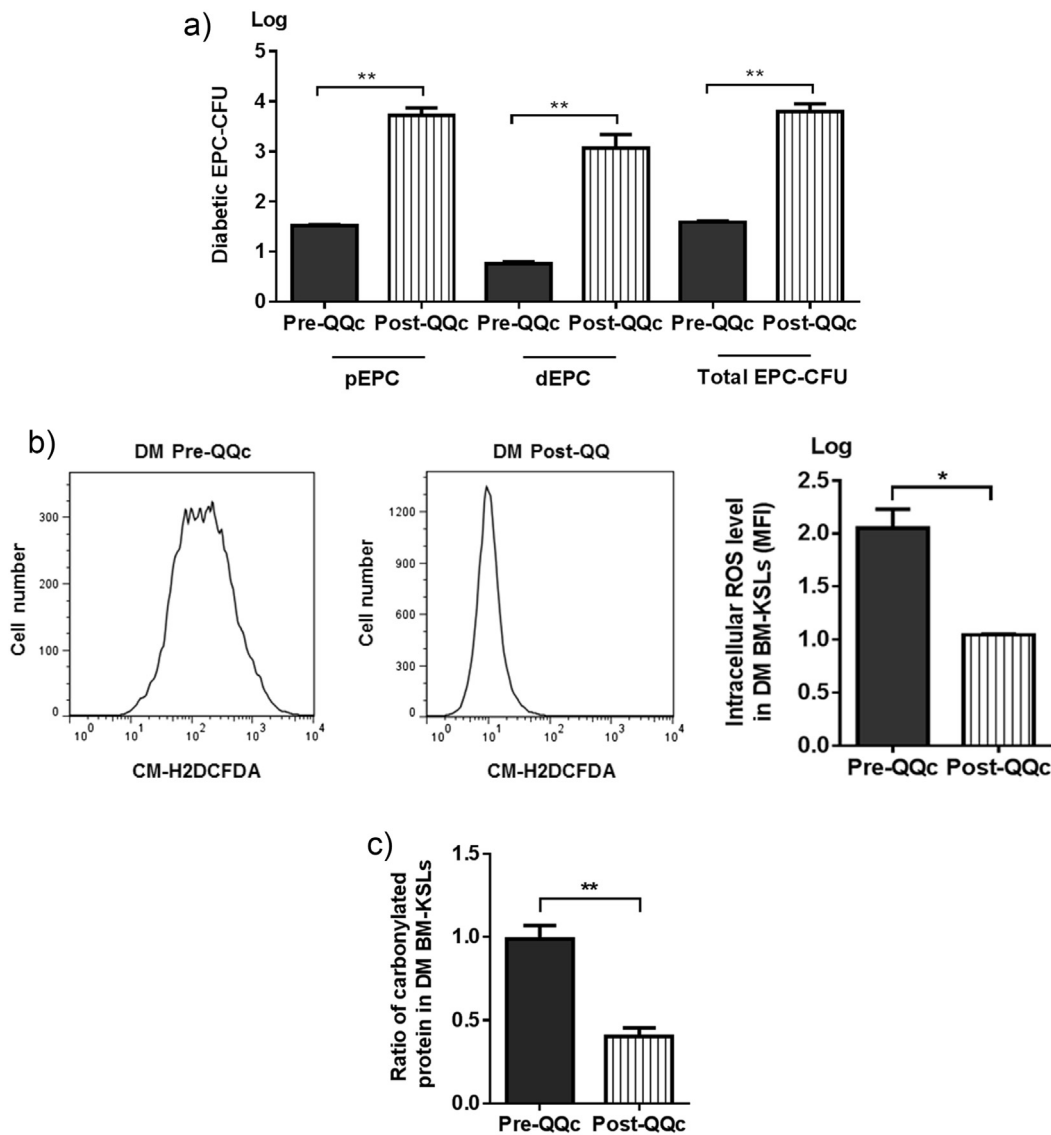
## 2.10. Statistical analysis

Comparison of the two groups with normally distributed variable was performed using student *t* test analyzed by GraphPad Prism 5. Statistical significance was defined as a value of  $p < 0.05$ . All data are presented as the mean  $\pm$  SEM. In this study the experiments were performed in three to five samples with two to three mice/sample.

## 3. Results

### 3.1. Diabetic BM-KSL is dysfunction and restored by QQc system

EPC vasculogenic function was assessed by EPC-CFU number [9,16,17]. DM BM-KSL have significantly lower number of dEPC-CFU ( $6.0 \pm 0.53$  vs.  $9.56 \pm 0.77$ ;  $p < 0.05$ ) and tEPC-CFU ( $39.33 \pm 1.93$  vs.



**Fig. 1.** Improvement of DM BM-KSL vasculogenic dysfunction by culturing in QQc system. a. Diabetic BM-KSLs dysfunction can be restored by culturing in QQc system (post-QQc) which showed increased the frequency of all stages of EPC-CFU. DM BM-KSL post-QQc showed increase in primitive EPC-CFU (pEPC) ( $p < 0.0001$ ), definitive EPC-CFU (dEPC) ( $p < 0.0001$ ), and total EPC-CFU (tEPC) ( $p < 0.0001$ ), respectively compared to DM pre-QQc. b. Intracellular ROS level in DM BM-KSL. Post-QQc of DM BM-KSLs showed decrease in intracellular ROS level ( $p < 0.05$ ) compared to control. c. Ratio of carbonylated protein (DNPH) in DM BM-KSL. Post-QQc DM BM-KSLs showed decrease in carbonylated protein ( $p < 0.05$ ) compared to pre-QQc.

46.0 ± 1.17;  $p < 0.05$ ) compared to control BM-KSL. To investigate the key factor restoring vasculogenic dysfunction in diabetic EPC we used QQc culture system as previously described [9]. First we compared DM BM-KSL in pre-QQc (fresh BM-KSL) and post-QQc for EPC-CFU number to assess vasculogenic potential. DM post-QQc demonstrated significant increase in primitive EPC-CFU (pEPC) ( $3.72 \pm 0.15$  vs.  $1.52 \pm 0.01$ ,  $p < 0.0001$ ), definitive EPC-CFU (dEPC) ( $3.07 \pm 0.27$  vs.  $0.77 \pm 0.04$ ,  $p < 0.0001$ ), and total EPC-CFU (tEPC) ( $3.79 \pm 0.15$  vs.  $1.59 \pm 0.02$ ,  $p < 0.0001$ ), respectively compared to DM pre-QQc (Fig. 1a). To determine whether QQc restores DM BM-KSL vasculogenic function followed by changes in ROS level, we investigated intracellular ROS level in DM BM-KSL pre and post-QQc. Intracellular ROS level of DM BM-KSL post-QQc was decreased ( $1.05 \pm 0.01$ ) compared to DM BM-KSL pre-QQc ( $2.05 \pm 0.18$ ;  $p < 0.05$ ) (Fig. 1b). In addition we also verified the carbonylated protein in DM BM-KSL pre and post-QQc. DM BM-KSL post-QQc demonstrated significant decreased in carbonylated protein level compare to DM BM-KSL pre-QQc ( $0.4 \pm 0.05$  vs.  $0.987 \pm 0.08$ ,  $p < 0.05$ ) (Fig. 1c). Taken together our findings suggest that QQc system restored vasculogenic potential of DM BM-KSL followed by decreased in ROS level.

### 3.2. Oxidative stress levels are not higher in diabetic BM-KSLs compared to controls

We next investigate the oxidative stress levels of DM BM-KSLs compared to control. Interestingly intracellular ROS level was comparable between DM BM-KSL cells ( $2.05 \pm 0.18$ ) and control ( $1.95 \pm 0.17$ ;  $p > 0.05$ ) (Fig. 2a). The carbonylated protein level of DM BM-KSLs ( $0.987 \pm 0.08$ ) also in similar amount compared to controls ( $1.0 \pm 0.03$ ,  $p > 0.05$ ) (Fig. 2b). These findings suggest that oxidative stress on DM BM-KSL is not significantly different compared to control BM-KSL cells.

### 3.3. Diabetic BM-KSLs have higher expression of anti-oxidative genes compared to controls

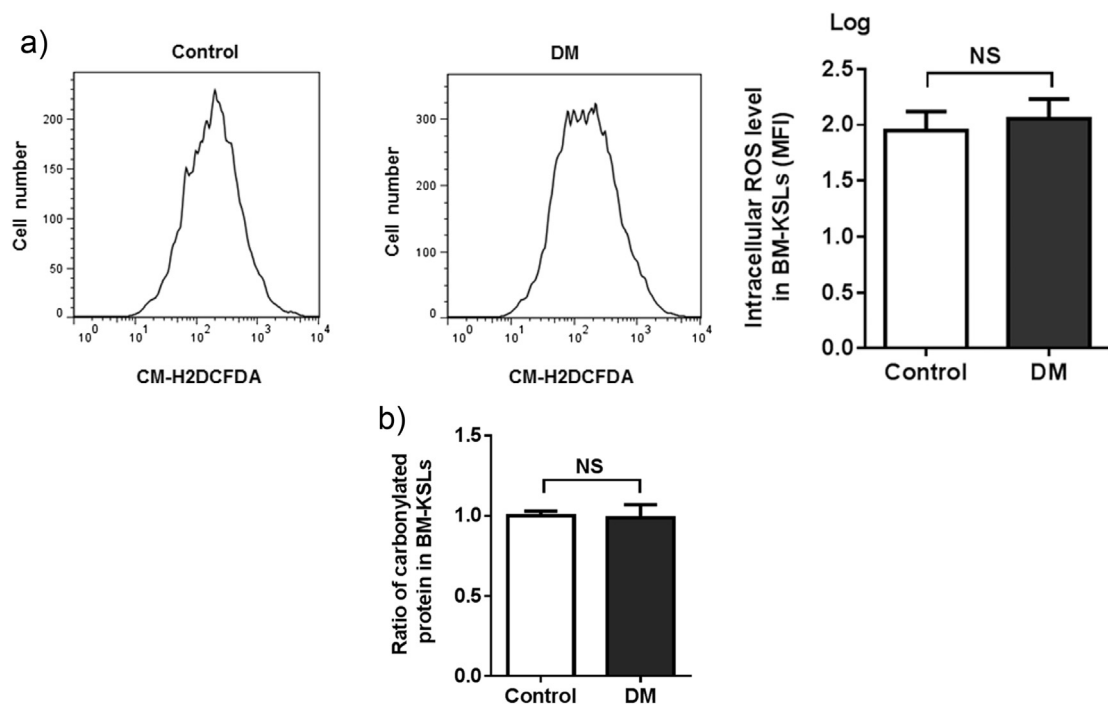
To screen the gene expression profile of BM-KSL cells for “cellular response to oxidative stress” (GO term 006979) we performed microarray analysis. A gene tree analysis of 363 probes related to “cellular response to oxidative stress” showed increase expressions of anti-oxidative genes such as superoxide dismutase (SOD), oxidative stress response1 protein (Oxsr1), peroxiredoxins (Prdx), GPx and catalase in DM BM-KSL cells (Fig. 3a) within 1.01 up to 1.36 fold increases. To assess the RNA expression of the main anti-oxidative enzymes we confirmed the results by RT-qPCR. DM BM-KSL showed higher expression of MnSOD (2.0 folds increase), GPx (3.49 folds increase) and catalase (2.2 folds increase) compared to control respectively (Fig. 3b). These results showed that there were augmented mRNA expressions of anti-oxidative enzymes in DM BM-KSL.

### 3.4. Diabetic BM-KSLs have higher activity of catalase compared to controls

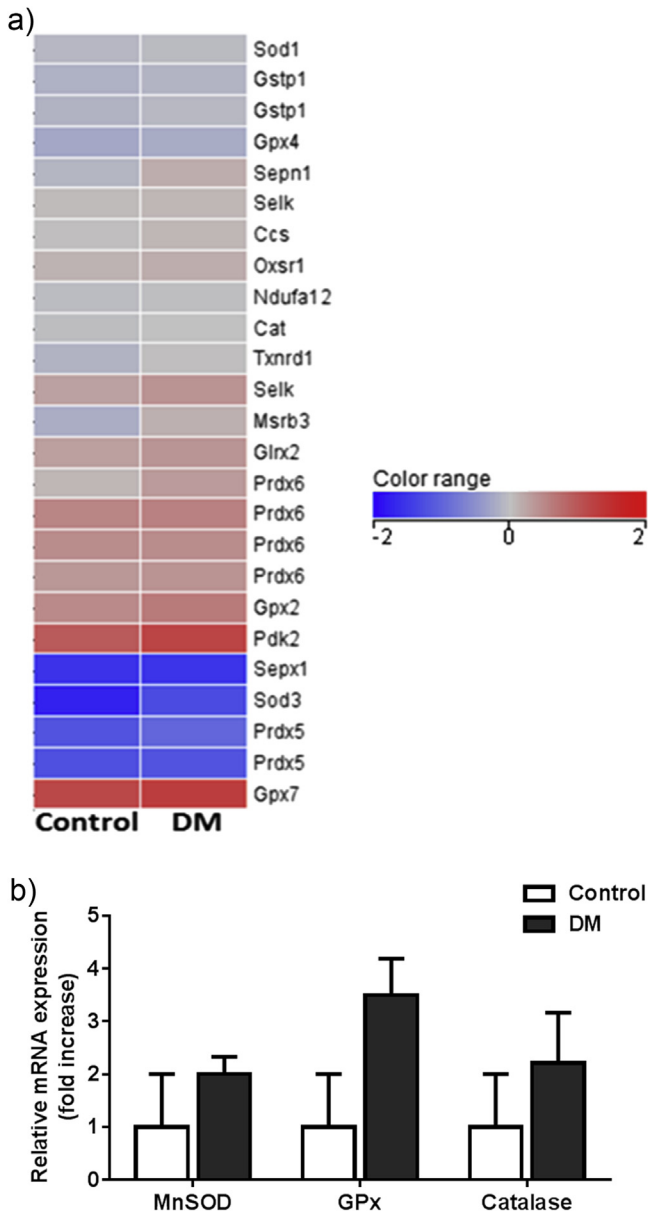
Catalase is an important enzyme in superoxide final removal through hydrogen peroxide detoxifying. DM BM-KSL showed slight increase in catalase activity ( $9.73 \pm 0.80$ ) compare to control ( $8.13 \pm 0.60$ ,  $p > 0.05$ ) (Fig. 4). Taken together with the results of anti-oxidative gene expressions, it indicates that DM BM-KSLs increase the catalase RNA expression followed by its enzyme activity to eliminate the oxidative stress received by the diabetic condition.

## 4. Discussion

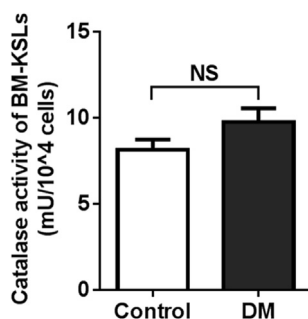
We investigated the vasculogenic potential of primitive BM-EPCs in the bone marrow hematopoietic population (KSLs) by EPC-



**Fig. 2.** Oxidative stress level in BM-KSLs. a. Intracellular ROS in DM BM-KSL was comparable to control and was not significantly different ( $p > 0.05$ ). b. Further measurement of ratio carbonylated protein in DM BM-KSL verified the result that oxidative stress in DM BM-KSL was not higher than control ( $p > 0.05$ ).



**Fig. 3.** Anti-oxidative genes expression in BM-KSL cells. a. Microarray analysis of BM-KSLs in “cell response to oxidative stress”. Expression levels are shown by color, blue representing low gene expression and red representing high expression. b. mRNA expression of anti-oxidative enzymes in BM-KSL. The figures represented fold increase in mRNA expression of the respective genes.



**Fig. 4.** Catalase activity of BM-KSL cells. DM BM-KSL cells showed slight increase in catalase activity compared to control and was not significantly different ( $p > 0.05$ ).

CFA and demonstrated that in early diabetic primitive BM-EPC has vasculogenic dysfunction in differentiative capacity as represented by lower definitive and total EPC-CFU number compare to controls. We further demonstrated that this vasculogenic dysfunction can be restored by culturing the BM-KSL cells in QQc system. Interestingly, compare to pre-QQc (freshly isolated BM-KSL cells), post-QQc DM BM-KSL cells not just improved their vasculogenic potential but also significantly decreased the oxidative stress of the cells. These data have addressed us that vasculogenic dysfunction of DM BM-KSLs could be restored by releasing the cells from diabetic oxidative stress environment and that oxidative stress may be the central cause of primitive EPC dysfunction in early diabetes.

Despite the fact of other studies reporting that EPC dysfunction is caused by oxidative stress [10–12,23], interestingly our result showed that DM BM-KSLs as primitive EPCs have similar oxidative stress level to control. Our results suggested that the oxidative stress is not greatly increased and may not be the strong factor to control the function of vasculogenesis in primitive DM BM-KSL cells.

There are several reasons to the discrepancy between our result and the other reports [24–27]. First, it may be due to the difference in cell source. We used freshly isolated BM-KSLs which represent as primitive BM-EPC, while others use circulating EPCs or cultured PB-MNC [10,11,24] or BM-MNC [27,28] which contains more differentiated EPC phenotype.

BM-KSLs reside in the bone marrow niche, a place of lowest end of physiological perfusion gradient with less blood perfusion providing lesser contact between the progenitor cells with harmful metabolites, we believe that the DM BM-KSLs receives less oxidative stress compared to circulating EPCs [29,30]. The second reason may be due to diabetic duration of the mice. Here we used 4th weeks STZ-induced diabetic mice. Although there is no study that directly compares the short and long term diabetes, study by Orlandi et al., has showed that bone marrow impairment was observed in long term diabetes (20 weeks) but not yet observed in short term diabetes (4–12 weeks) [31].

Another interesting finding of our study showed increase expression of MnSOD, GPx and Catalase in DM BM-KSL compare to control. Taken together with the results of oxidative stress levels, our results indicate the possible existence of compensatory mechanism in DM BM-KSLs to fight against oxidative stress by increasing the expression and activity of anti-oxidative genes [32–34]. This finding is in line with the previous studies which demonstrated higher anti-oxidative gene expression in progenitor cells [32–34]. All together, we believe that level of oxidative stress is well tolerated and not at the level where diabetic BM-KSLs is damaged to exhibit high level of ROS and carbonylated protein and to affect the vasculogenic potential. Therefore, the vasculogenic dysfunction of early stage DM BM-KSLs may happen before the cells are receiving oxidative stress, under other mechanism of oxidative stress mediating in cellular damage/to cause dysfunction. It also indicates the possibilities of other underlying mechanism which is activated earlier than oxidative stress in mediating cellular damage/dysfunction. Since Notch pathway has been shown to have close relation to EPC functions such proliferation, adhesion, apoptosis [35], mobilization [36] and play important role in EPCs development [37,38] we are now investigating the possibility of Notch signaling as one of the cause of DM BM-KSLs dysfunction.

## 5. Conclusion

Our study have demonstrated for the first time that oxidative stress level in early diabetic is not the main factor taking in part of primitive bone marrow EPC dysfunction. Our findings indicate that diabetic BM-KSLs demonstrate to withstand towards oxidative

stress in part through increasing the expression and activity of anti-oxidative genes. In addition, there is possibility that oxidative stress is not the initiating factor of vasculogenic dysfunction in diabetic primitive BM-KSL. Our study has provided new insights to further elucidate the possible mechanism which is activated prior to oxidative stress in causing vasculogenic impairment of DM BM-KSL.

### Author contributions

**DS:** conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing. **SF:** collection and assembly of data, data analysis and interpretation, manuscript editing. **SJ:** collection and assembly of data. **RIH:** collection and assembly of data. **TI:** conception and data interpretation. **TS:** collection and assembly of data. **AH:** manuscript editing and final approval of manuscript. **SI:** final approval of manuscript. **HM:** final approval of manuscript. **HD:** final approval of manuscript. **RT:** conception and design, data interpretation, manuscript writing, final approval of manuscript and financial support.

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### Conflict of interest

All the listed authors: Dewi Sukmawati, Satoshi Fujimura, Sachie Jitsukawa, Rie Ito-Hirano, Takamasa Ishii, Tadayuki Sato, Ayato Hayashi, Seigo Itoh, Hiroshi Mizuno, Hiroyuki Daida and Rica Tanaka declare that they have no conflict of interest regarding this study.

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