

Hematopoietic recovery following chemotherapy is improved by BADGE-induced inhibition of adipogenesis

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Abstract This study was designed to investigate the role of increased adipocytes in the bone marrow (BM) niche induced by high-dose chemotherapy in hematopoietic recovery. Arabinosylcytosine (Ara-C) was administered to adult C57BL/6J mice to induce adipogenesis in the BM. We investigated the effects of adipogenesis on hematopoietic recovery following chemotherapy, using the peroxisome proliferator-activated receptor gamma inhibitor, bisphenol A diglycidyl ether (BADGE). Adipocyte hyperplasia could be induced by Ara-C treatment in BM and inhibited by BADGE. The accelerated recovery of leukocyte counts, increased colony forming units, and a higher proportion of Ki67⁺CD45⁺ BM cells and Ki67⁺Lin⁻Sca1⁺c-kit⁺ hematopoietic stem cells were observed in the

long bone marrow of adipocyte-inhibited mice, as well as an increase in the number of CD45⁺ BM cells in the tail fatty marrow compared to controls. Adipocytes participated in creating a distinctive niche for hematopoietic cells. In addition, lower expression of stromal cell-derived factor-1 α and hypoxia-inducible factor-1 alpha were detected in the BADGE-treated group. These results indicate that hematopoietic recovery is improved following chemotherapy in adipogenesis-inhibited mice. In addition, adipocytes may create an individual niche that affects the proliferation and migration of hematopoietic cells in vitro and in vivo.

Keywords Adipocyte · PPAR γ inhibitor · Hematopoietic recovery · Bone marrow niche

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Introduction

The bone marrow (BM) hematopoietic microenvironment, which represents an important niche for hematopoietic stem cells (HSCs), is composed of extracellular matrix as well as complex cellular components including osteoblasts, endothelial cells, mesenchymal progenitor cells and adipocytes (commonly referred to as 'fat cells'). Compared with other cellular components, the role of adipocytes in the hematopoietic niche are much less concerned [1].

The number of adipocytes varies in the hematopoietic niche under different physiologic and pathologic conditions [2–4]. Adipocyte hyperplasia, in addition to hematopoietic hypocellularity in the BM, is a pathologic characteristic observed in clinical hematological disorders. One of the pathologic conditions leading to an increase in the fat fraction in the BM microenvironment is high-dose chemotherapy. Hematopoiesis may be either partially hypoplastic or almost completely absent (aplastic), and is replaced by

extended fatty tissue induced by effective and aggressive drugs [5–10]. The hyperplastic adipocytes induced by chemotherapy are always considered to play a passive role by occupying BM space [1]. However, recent studies reveal that adipocytes may play a negative role in the BM. For example, marrow engraftment is accelerated in A-ZIP/F1 mice which do not contain fat cells in BM under normal condition, relative to wild-type mice [11]. However, the influence of adipocytes on hematopoietic recovery after chemotherapy has not been elucidated. How adipocytes hyperplasia disturb the BM niche remains unknown.

The peroxisome proliferator-activated receptor gamma (PPAR γ), is a member of the nuclear-receptor superfamily, and is a vital transcriptional regulator of fat formation. To date, no factors capable of regulating adipogenesis independently of PPAR γ have been identified [12]. Both isoforms of PPAR γ , PPAR γ -1 and PPAR γ -2, are expressed at high levels during adipogenesis, with PPAR γ -2 being the predominant isoform found in adipose tissue [13–15]. Bisphenol A diglycidyl ether (BADGE) is a synthetic antagonist of PPAR γ and has previously been shown to prevent adipogenesis of the pre-adipocyte line 3T3-L1, and bone marrow adipocyte formation after irradiation in murine models [11, 16, 17]. Whether adipogenesis in the BM induced by chemotherapy agents can be prevented by BADGE, has not yet been reported.

Arabinosylcytosine (Ara-C) is an anti-cancer chemotherapy drug. As an anti-metabolite, it is commonly used to treat different types of leukemia.

The aim of this study is to develop an adipocyte hyperplasia model by treatment of mice with Ara-C, and to investigate the effects of the PPAR γ inhibitor, BADGE, on Ara-C-induced adipogenesis. To investigate the influence of adipocyte hyperplasia on hematopoietic recovery, peripheral blood analysis was performed and the proliferative capacity of bone marrow cells in both long bone marrow and the tail vertebrae marrow was assessed. Meanwhile, the expression of HIF-1 α (hypoxia-inducible factor-1 alpha) and SDF-1 α (stromal cell-derived factor-1 α) was detected to elucidate the effect of adipocyte in the BM niche.

Materials and methods

Mice

C57BL/6J female mice (6–8 weeks old) were obtained from the Experimental Animal Center of Military Medical Sciences Academy (Beijing, China). Mice were housed in a controlled environment (12 h light/dark cycles at 21 °C). Animal experiments were performed with authorization from the Animal Ethics Committee of Peking University Health Science Center.

Treatment of mice

For induction of hematopoietic stress, adult mice (~20 g, $n = 70$) were administered 0.5 g/kg Ara-C (Sigma, USA) via intraperitoneal injection, once daily for four consecutive days, in accordance with the previous reports [18].

For PPAR γ inhibitor treatment, 60 mg/kg BADGE (Sigma, USA) was administered daily via intraperitoneal injection into mice ($n = 35$), from day 0 until 4 weeks after Ara-C treatment. Control group animals ($n = 35$) were injected with 10 % DMSO (Sigma, USA). BADGE was prepared as previously described [11].

To be a positive contrast, 300 μ g/kg mouse G-CSF (Sigma, USA) was administrated subcutaneously to mice ($n = 25$) once a day for 5 days post Ara-C treatment.

Peripheral blood analysis

Retro-orbital peripheral blood was collected at weekly intervals following Ara-C treatment using microhematocrit capillary tubes (Fisher Scientific, UK). Leukocyte and platelet counts and hemoglobin levels were analyzed using a hematology analyzer Hemavet Model HV950 (Drew Scientific, UK).

Bone marrow extraction

Mice from BADGE and control groups were sacrificed between 1 and 4 weeks following Ara-C treatment. Femurs, tibias and tail vertebrae were harvested, and bones were crushed with a scalpel in PBS containing 2 % (v/v) fetal bovine serum (FBS, GIBCOTM, USA) and 1 mM EDTA. Suspensions were digested with 3 mg/ml Collagenase I (Worthington, USA) for 30 min, filtered through a 70- μ m nylon filter cell strainer (BD, USA) and washed with PBS.

Cell culture and adipogenic differentiation

For marrow stromal cell culture, cells harvested from the BM of 6 weeks old C57BL/6J mice were plated in T25 flasks (Corning, USA) in MesenCult[®] MSC Basal medium with supplement medium (Stem Cell, Canada), penicillin and streptomycin, and cultured at 37 °C with 5 % CO₂. Adherent stromal cells were cultured to 85 % confluence. After trypsinization with 0.25 % (m/v) Trypsin–EDTA (GIBCOTM, USA), they were collected and cultured for 2 passages to obtain bone marrow stromal progenitor cells (MPCs). MPCs were maintained in 6-well plates (Costar, USA). At passage three, cells were cultured with Adipogenic Differentiation Medium according to the manufacturer's instructions (Cyagen OricellTM, China) and then treated with 0.3 % DMSO or BADGE (20 and 100 μ M), commencing on the first day of adipogenic differentiation.

Adipogenesis was determined by staining lipids with Oil Red O (Cyagen Oricell™, China), according to the manufacturer's instructions. The pre-adipocyte line 3T3-L1 cells were cultured in the same medium as MPCs and then adipogenic differentiated with the similar procedures.

Hematopoietic cell isolation

BM cells from femurs and tibias were pooled, and hematopoietic progenitors were isolated by depleting lineage positive cells using magnetic active cell sorting (MACS, Negative Selection Mouse Hematopoietic Progenitor Cell Enrichment Kit, Stem Cell, Canada) according to the manufacturer's instructions. Cells were labeled with a cocktail of rat anti-mouse antibodies including B220 (CD45R), Mac-1 (CD11b), GR-1 (Ly-6G), CD5, CD19 and Ter119. Lineage negative (Lin⁻) cells were obtained using anti-rat beads, and cells were stained with antibodies for flow cytometric analysis.

Co-culture (contact) experiments

MPCs (passage 3) were plated in 24-well plates and cultured in adipogenic differentiation medium as described above. Cells were treated with 0.3 % DMSO (control) or BADGE (20 and 100 μM), commencing on first day of adipogenic differentiation. After 7 days, when differentiation had occurred, 1×10^5 Lin⁻ BM cells were added to each well and co-cultured with the BM stromal progenitors, which included varying numbers of adipocytes, for 3 days. As an additional control, MPCs were co-cultured with the same number of Lin⁻ BM cells in the absence of adipogenic differentiation agents. Adipocytes derived from MPC co-culture experiments were determined by Oil Red O staining. After co-cultured, Lin⁻ cells and their derived cells were blown down gently and then collected.

Migration experiment (non-contact co-culture)

In the migration system (5 μm pore size of filter, Coster, USA), MPCs were cultured in adipogenic differentiation medium and treated with 0.3 % DMSO (control) or BADGE (100 μM) in the lower chambers of 24 wells. Then 1×10^5 Lin⁻ BM cells were placed into the top chambers of 24 wells. After 72 h, the Lin⁻ BM cells migrated adhering to the lower surface of the filter were stained with Wright's staining and their number was counted.

Cell proliferation analysis

MPCs (2×10^4 /well) were plated in 96-well plates. After 24 h, 0.3 % DMSO (control) or BADGE (25, 50, 100 and 200 μM) was added to the wells and cells were cultured for

3 days. Cell proliferation was analyzed using a Cell Counting Kit-8 (CCK-8, Dojindo, Japan) according to the manufacturer's instructions.

Progenitor cell assay

Colony forming units (CFUs) were analyzed by plating 5×10^4 BM cells in 35-mm petri dishes containing 1 ml of methylcellulose with cytokines (MethoCult GF M3434, Stem Cell, Canada). Cultures were maintained at 37 °C with 5 % CO₂. Colonies were counted after 8–10 days using an inverted microscope (Olympus, Japan).

Histopathology

Mice were sacrificed using ether anesthesia, and tibias and the fifth to sixth segments of tail vertebrae were collected and fixed in 4 % (w/v) paraformaldehyde for 24 h. Tissues were decalcified in 20 % (w/v) EDTA (pH 7.5) for 7 days at 4 °C, and paraffin embedded. Sections (4 μm thick) were mounted on slides and deparaffinized. Then they were stained with hematoxylin and eosin (HE).

ELISA

Peripheral blood serum sample and supernatant were obtained, respectively, from different groups of mice and co-culture systems. Murine G-CSF and SDF-1α levels were determined by standard sandwich ELISA, according to the instructions of the manufacturer (R&D Systems, USA). Optical density was measured using a microplate reader set to 450 nm with correction at 570 nm.

Flow cytometry analysis

BM cells or Lin⁻ BM cells were harvested, washed and resuspended in PBS containing 2 % (v/v) FBS. Cells were stained with CD45-FITC (1:100, BD, USA), c-Kit-PerCP (1:200, Biolegend), Sca1-APC (1:100, Biolegend, USA), and corresponding isotype controls for 30 min at 4 °C. For cell cycle analysis, cells were first stained for CD45, c-Kit and Sca1 for 15 min, and subsequently washed and treated using the BD Intrasure™ kit. Cells were then washed and incubated with Ki-67-FITC (1:100, Abcam, UK) or isotype control for 20 min at 4 °C. Cells were washed, resuspended in PBS containing 2 % (v/v) FBS and analyzed on a FACSCalibur flow cytometer (BD, USA). Data acquisition and analysis were performed using Express 3 software.

Quantitative real-time PCR (qPCR)

Total RNA was isolated from pooled marrow cells using TRIzol Reagent (Invitrogen, USA). 1 μg of RNA was

Table 1 Primer pair sequences used in RT-PCR analysis

aP2 forward	TGTGCGAAACTGAATTCCTGC
aP2 reverse	GAGATCGGTCTGAGCCAGC
PPAR γ -2 forward	ACAGGAAAGACAACGGACAAATC
PPAR γ -2 reverse	GGATCGAAACTGGCACCCCT
Adiponectin forward	GCACACCAGGCCGTGATG
Adiponectin reverse	CCTTTCCTGCCAGGGGTTC
Neuropilin-1 forward	CTGTGCAAAACCAACAGACCTAG
Neuropilin-1 reverse	AGCACATTGCCTGGCTTCC
HIF-1 α forward	TGAACAGAAATGGAACGGAGCA
HIF-1 α reverse	TGCTGCCTTGTATGGGAGC
SDF-1 α forward	AGATGCCTTGTCTGAGTC
SDF-1 α reverse	AGGTGAGAAGCGGAAGTC
GAPDH forward	TCAATGACAACCTTTGTCAAGCTCA
GAPDH reverse	GTGGGTGGTCCAGGGTTTCTTACT

reverse transcribed using a HiFi-MMLV cDNA Kit (Cwbio, China). qPCR was performed in a final volume of 20 μ l comprising 2 μ g of cDNA mixed with optimal concentrations of primers and probe and UltraSYBR Mixture (with Rox) (Cwbio, China) in 96-well plates. Reactions were performed using a 7500 Fast Real-Time PCR System (Life Technologies, USA). Primer and probe sets were designed using Primer 5.0 software (Table 1). Amplification was performed as follows: 10 min at 95 $^{\circ}$ C, then 40 cycles of 15 s at 95 $^{\circ}$ C and 60 s at 60 $^{\circ}$ C. Murine *GAPDH* was amplified as an endogenous control. Relative quantitation was calculated using the $2^{-\Delta\Delta C_T}$ method.

Statistics

Data are expressed as the mean \pm standard deviation. Statistical differences between two groups were evaluated by Student's *t* test. For multiple group comparisons, data were analyzed by one-way analysis of variance (ANOVA). Differences were considered significant when *p* value was below 0.05.

Results

Development of an adipocyte hyperplasia marrow model under hematopoietic stress by Ara-C

MPCs can be differentiated into adipocytes and osteoblasts in vitro [19, 20]. We investigated whether BADGE effectively inhibits adipogenesis of BM stromal progenitors in vitro, similar to previous reports [17] (Supplemental Figure 1). We next developed an adipocyte hyperplasia marrow model, by inducing hematopoietic stress in mice by treatment with Ara-C. Bone marrow from normal, 6–8 weeks adult mice showed active hematopoiesis, with

few adipocytes and abundant normal sinuses (Fig. 1a). However, after 1 week of treatment with Ara-C (2.0 g/kg), mice exhibited hypoplastic hematopoiesis and hemorrhage. In comparison to normal BM sections, the sinuses of treated mice were widely dilated, hyperemic and composed of discontinuous endothelial cells. Hyperplasia of adipocytes were observed in the metaphysis around the sinuses or attached to the sinus walls (Fig. 1b). A significant increase of adipocyte counts was also observed in the tibias of Ara-C-treated mice (Fig. 1c). In addition, the expression of adipokines *PPAR γ -2* and *aP2* in the marrow of long bones was increased following chemotherapy treatment, compared with normal healthy mice (Fig. 1d).

In contrast to the long bone marrow, marrow from the tail vertebrae of normal healthy 8-week mice was filled with adipocytes, and contained few hematopoietic cells, in addition to several normal sinuses (Fig. 1e). After 1 week of Ara-C treatment, tail vertebrae BM sections also showed widely dilated sinuses around the endosteum, predominantly in the metaphysis (Fig. 1f). While no remarkable increase in adipocytes was observed, the expression of *PPAR γ -2* and *aP2* was elevated following Ara-C treatment (Fig. 1g).

Adipogenesis in both of long bones and tail vertebrae BM following Ara-C treatment is inhibited by BADGE

We next investigated the effect of BADGE on adipogenesis in mice treated with Ara-C. No significant histopathological differences between the two groups of mice (treated with BADGE and DMSO) were observed in the first week following Ara-C treatment (data not shown). However, at 2 and 3 weeks post Ara-C treatment, decreased numbers of adipocytes were observed in the long bone marrow of BADGE-treated mice, compared to control (Fig. 2a), though the difference shown minor at week 4 (data not shown). This result was further confirmed by adipocyte density analysis in marrow sections (Fig. 2c). By the way, BADGE did not show effects on adipogenesis of BM in normal and healthy mice (Fig. 2b).

In addition, we also observed a 10 % decrease in the weight of mice treated with BADGE, compared to control-treated mice (Fig. 2e), and these mice also displayed a thinner subcutaneous adipose layer in the abdominal wall (data not shown).

Since BADGE successfully inhibited adipocyte proliferation, we next asked whether this compound also affected the expression of adipokines. Both *aP2* and *adiponectin* (*Adipoq*) are specifically expressed in adipocytes [21, 22], and *neuropilin-1* (*Nrp1*) has been shown to be expressed at higher levels in the fatty marrow compared to the hematopoietic marrow [23]. Thus, we examined the expression of these genes in addition to *PPAR γ -2* by qRT-PCR. As expected, we found that the expression of all four genes

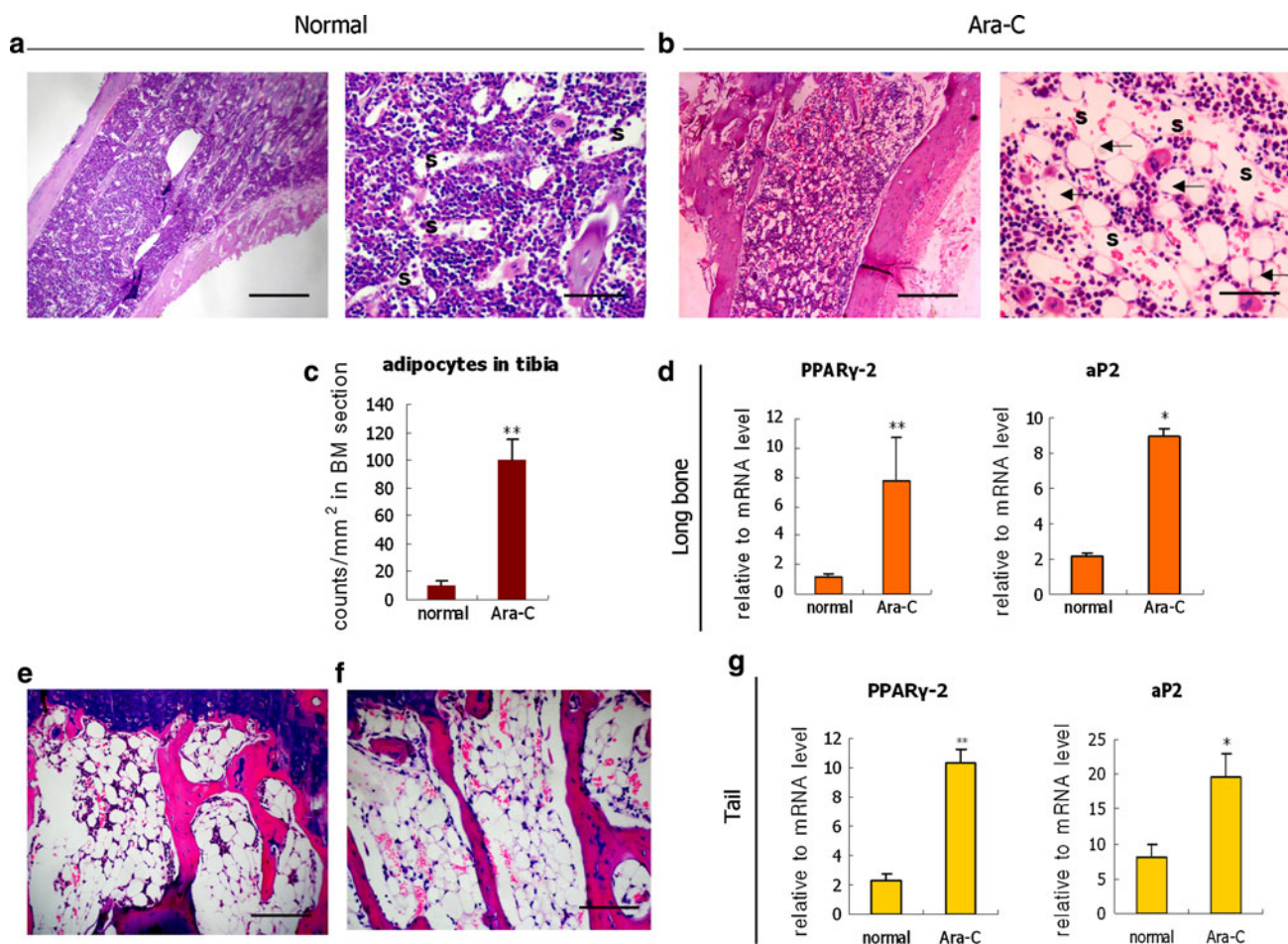


Fig. 1 **a** BM sections of the proximal tibia from 8-week-old normal mice (left, scale bar 400 μ m; right, scale bar 100 μ m, HE staining). **b** BM sections of the tibia 1 week after Ara-C treatment (left, scale bar 400 μ m; right, scale bar 100 μ m, HE staining). **c** Adipocyte counts per mm² in tibia marrow sections from both normal and Ara-C-treated mice. **d** Expression of *PPAR* γ -2 and *aP2* in the long bone

BM from Ara-C-treated mice. **e** Tail marrow sections from 8-week-old normal mice and **f** 1 week after Ara-C treatment (scale bar 200 μ m, HE staining). **g** Expression of *PPAR* γ -2 and *aP2* in tail vertebrae marrow of Ara-C-treated mice. Bars represent the mean \pm SD, * p < 0.01, ** p < 0.05, n = 6 mice; black arrow adipocyte, S sinus

was suppressed by treatment with BADGE at week 2 in the long bone marrow (femurs and tibias). This difference was less dramatic at week 3, which may be due to decreased adipocytes observed at this time point, following hematopoietic recovery (Fig. 2f).

Similarly, we observed a decrease in the number of adipocytes in the tail BM of BADGE-treated mice at weeks 2 and 3, compared to control, although this effect was no longer observed at week 4 (Fig. 2d, pathologic section shown in Fig. 5a). In addition, the expression of adipocyte-specific genes was reduced in BADGE-treated mice at weeks 2 and 3 (Fig. 2g).

Peripheral leukocyte counts are enhanced in BADGE-treated mice under hematopoietic stress

To investigate the effect of *PPAR* γ inhibitor on hematopoietic recovery, peripheral blood was analyzed in mice

following Ara-C and BADGE treatment, as well as the normal healthy mice treated with BADGE directly. Peripheral white blood cell (WBC) counts of BADGE and control-treated mice declined on day 3 post Ara-C treatment. However, WBC counts were elevated in BADGE-treated mice commencing at week one until week 4, compared to control mice (treated with DMSO) and normal healthy mice treated with only BADGE (Fig. 3a). Furthermore, we also observed a significant increase in neutrophils in BADGE-treated mice after chemotherapy, compared to control (Fig. 3b). While the hemoglobin (HB) content was not affected by treatment with BADGE, we observed a trend toward increased platelets compared to controls (Fig. 3c, d). We observed no increase in peripheral blood after BADGE treatment (Fig. 3a–d). G-CSF was an effective agent for stimulating progenitors to proliferate into leukocytes. To investigate the reason for improved hematopoietic recovery in adipocyte-inhibited group mice,

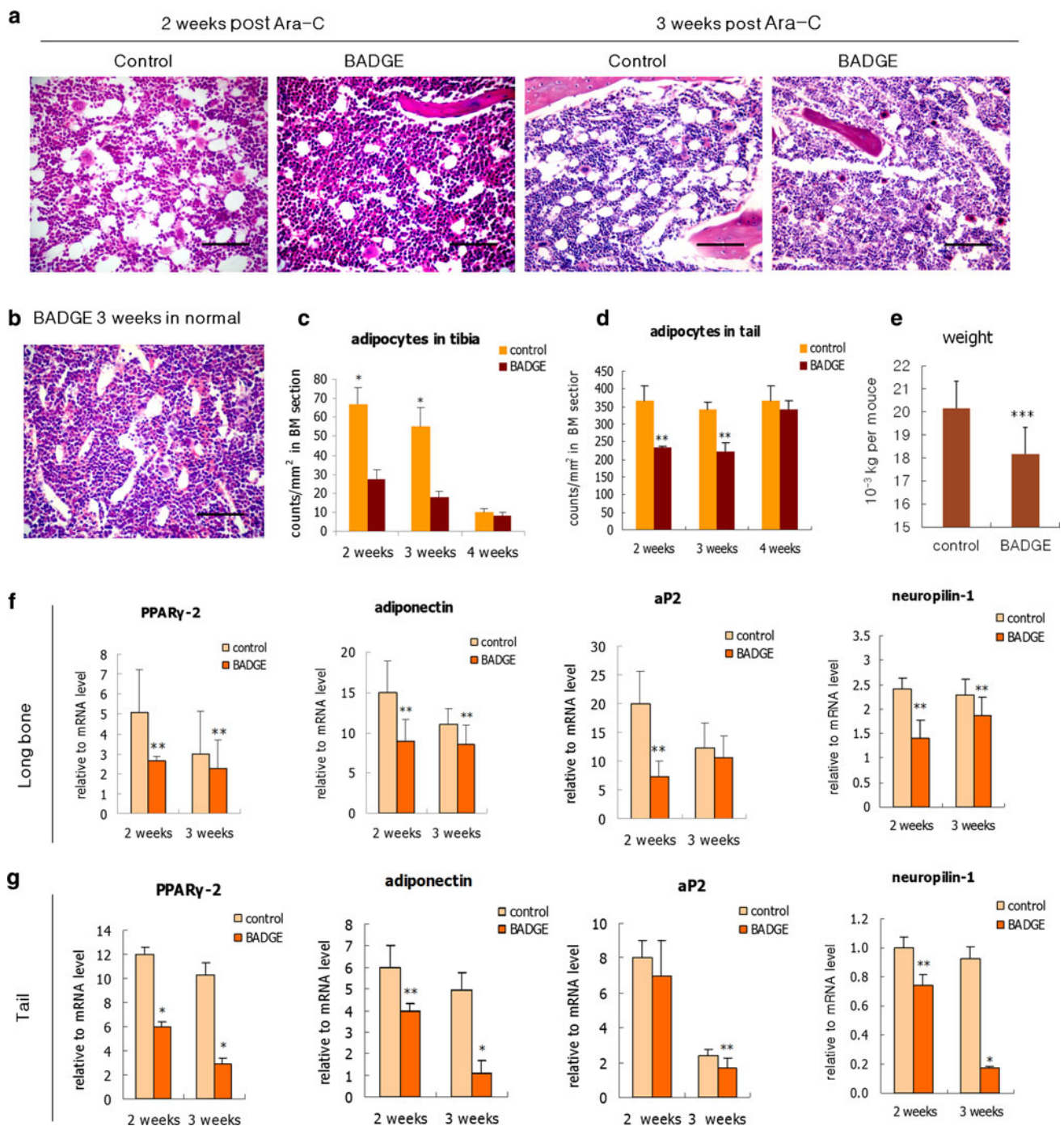


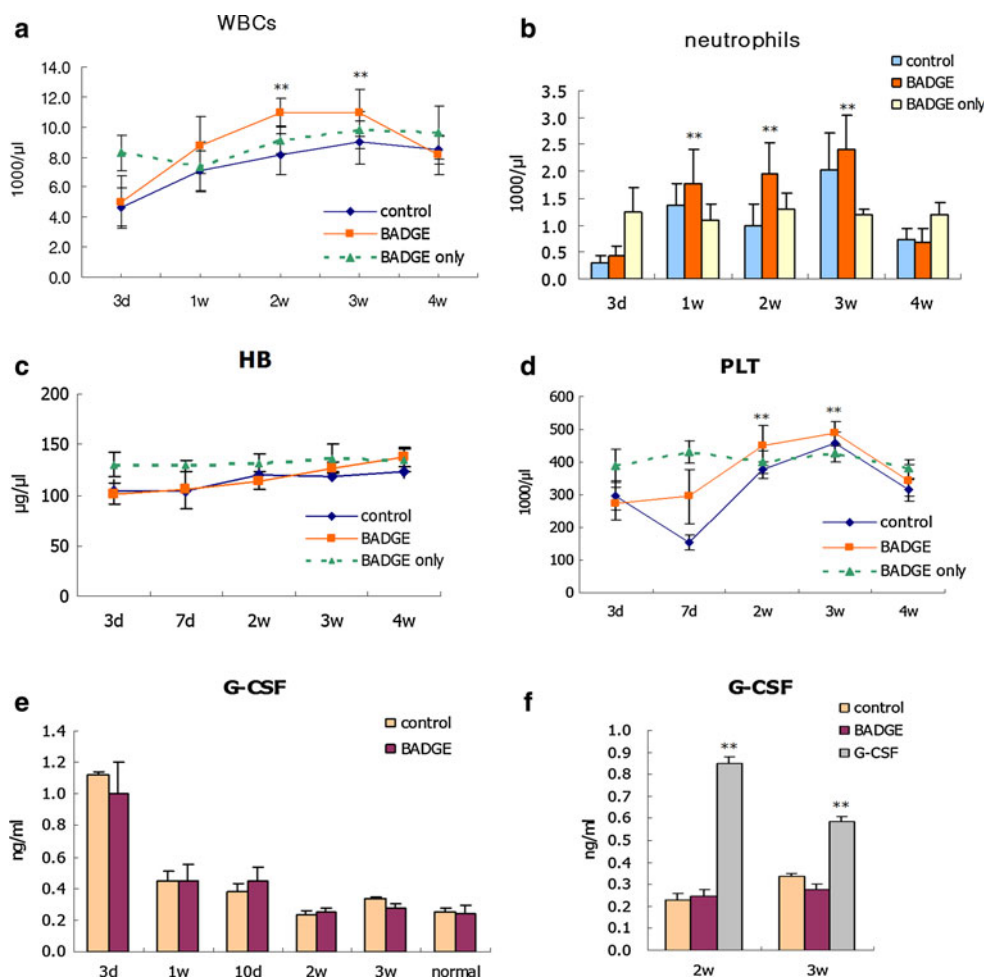
Fig. 2 **a** Adipocytes in BM sections of tibias from BADGE-treated mice and the controls following Ara-C treatment (HE staining, scale bar 100 μ m). **b** BM sections of tibias from BADGE-treated mice without Ara-C treatment (HE staining, scale bar 100 μ m). **c** Adipocyte counts per mm² in tibia BM sections and **d** tail BM sections from two groups of mice post Ara-C treatment. **e** A significant decrease in

weight was observed in BADGE-treated mice, compared with the control group. **f** Expression of *PPAR γ -2*, *aP2*, *Adpoq* and *Nrp1* in the long bone BM and **g** tail BM of two groups of mice after Ara-C treatment. Data represent the mean \pm SD, $n = 6$ in each group of mice, * $p < 0.01$, ** $p < 0.05$, *** $p < 0.02$

serum G-CSF level was detected which showed no difference between the two groups. We investigated that G-CSF level in peripheral blood of BADGE-treated group and DMSO-treated group enhanced on day 3

post-chemotherapy, but declined along with time (Fig. 3e). And the levels had no difference between the two groups, contrasting to a significantly G-CSF increase in the positive control group on weeks 2 and 3 (Fig. 3f). In addition,

Fig. 3 **a** A significantly earlier recovery of white blood cells (WBCs) and **b** a higher number of neutrophils was observed in BADGE-treated mice post Ara-C treatment compared to control mice and the normal mice treated with BADGE only. **c** No significant difference in hemoglobin levels was observed in BADGE-treated compared to control mice and the normal mice treated with only BADGE. **d** A trend toward increased platelets was observed in BADGE-treated compared to control mice and the normal mice treated with only BADGE. **e** Serum G-CSF level in peripheral blood showed no difference between BADGE-treated group and DMSO-treated group. BADGE did not enhance G-CSF level in normal mice ($p > 0.05$). **f** A significantly G-CSF increase in the positive control group on weeks 2 and 3, compared with BADGE-treated group and DMSO-treated group. Data represent the mean \pm SD, $n = 8$ in **a–d**, $n = 6$ in **e–f**; $**p < 0.05$



BADGE did not enhance G-CSF level in normal healthy mice (Fig. 3e).

Proliferation of hematopoietic cells in the long bone marrow is improved in BADGE-treated mice

Hematopoietic CFU assays reflect the number and proliferative capacity of hematopoietic progenitors in vitro. We observed an increase in CFUs in BM cells from BADGE-treated mice compared to controls at weeks 2 and 3 post Ara-C treatment; however, this effect was not detected at week 4 and BADGE showed no effect on increase of CFU in normal healthy mice (Fig. 4a).

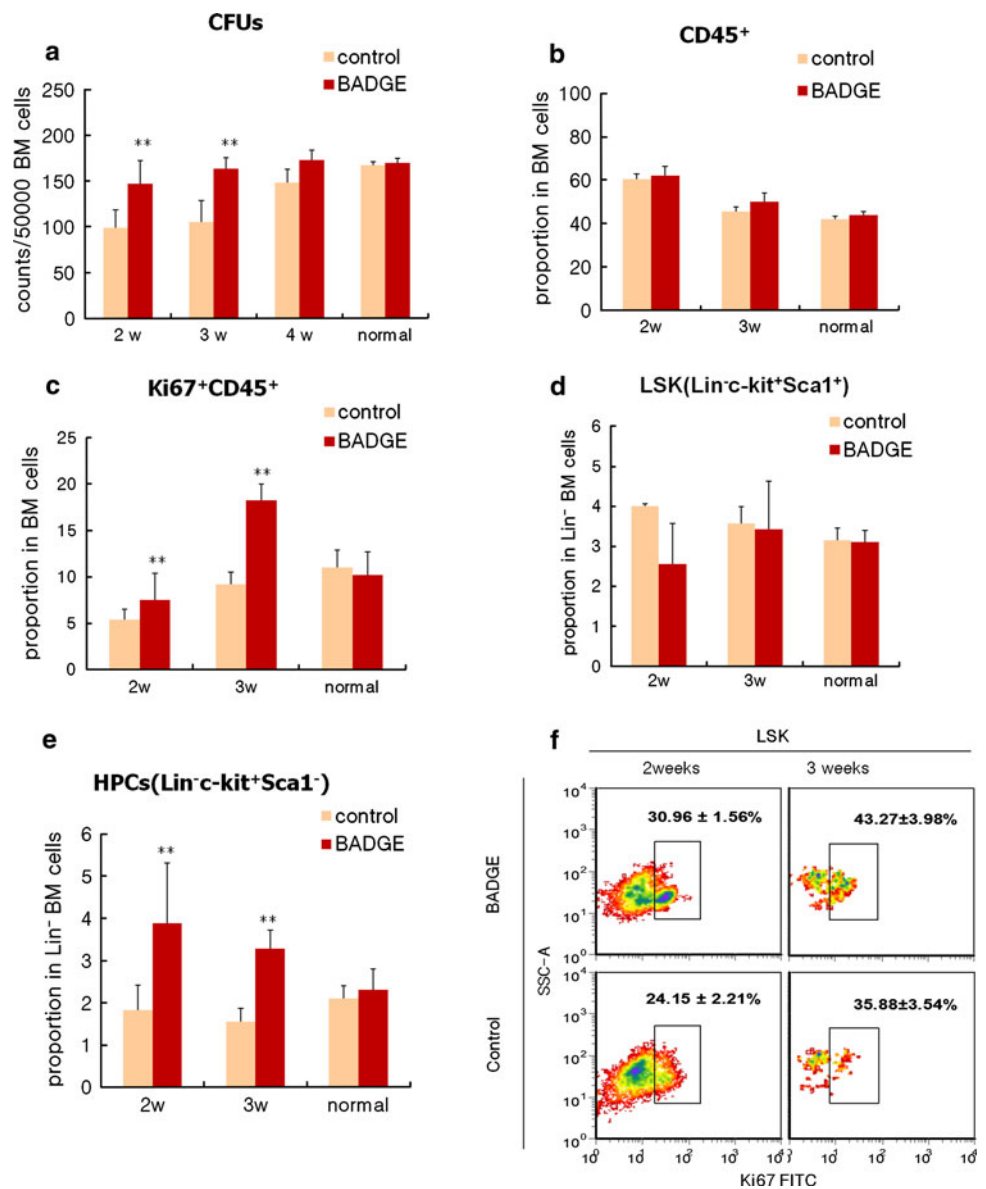
Based on the results of the peripheral blood analysis, we next compared the proliferative capacity of BM cells from BADGE and control mice at weeks 2 and 3 following Ara-C treatment. Ki-67 is a cell cycle-associated protein present during active phases of the cell cycle (G_1 , S, G_2 and mitosis), but is absent from resting cells (G_0), thus serving as a marker of proliferative cells [24]. Flow cytometric analysis revealed no significant difference in the proportion of CD45+ cells in BADGE-treated mice compared to controls

(Fig. 4b). In contrast, the proportion of CD45⁺ Ki67⁺ BM cells was significantly increased in BADGE-treated mice at 2 and 3 weeks compared to control (Fig. 4c). The frequency and cycling status of HSCs in these two groups was also analyzed. This analysis revealed that the number of Lin⁻ Sca1⁺c-kit⁺ (LSK) HSCs between BADGE and control-treated mice was similar (Fig. 4d). However, as expected, Ki67 expression was increased in LSKs from BADGE-treated mice compared with the controls (Fig. 4f). BADGE also showed no effect on hematopoietic proliferation in normal healthy mice (Fig. 4b–e). In addition, the proportion of Lin⁻ Sca1⁻c-kit⁺ hematopoietic progenitor cells (HPCs) was increased compared with controls (Fig. 4e).

Hematopoietic cell infiltration in tail vertebrae BM is improved in BADGE-treated mice

Since hematopoietic cell proliferation in the long BM was facilitated in BADGE-treated mice, we next investigated the effect of treatment on the tail marrow, which is filled with abundant fat cells during homeostasis in adult mice. Intriguingly, we observed a remarkable infiltration of

Fig. 4 **a** Colony forming units (CFUs) in BADGE-treated and control mice after Ara-C treatment ($n = 8$). **b** No significant difference in the proportion of CD45⁺ BM cells were observed between the two groups ($n = 6$, $p > 0.05$). **c** Proportion of Ki67⁺CD45⁺ BM cells in BADGE-treated and control mice ($n = 6$). **d** No significant differences in the proportion of Lin⁻Sca1⁺c-kit⁺ (LSK) HSCs in Lin⁻ BM cells was observed between BADGE-treated and control mice ($n = 6$, $p > 0.05$). **e** Proportions of Ki67⁺ LSK HSCs in mice treated with BADGE and the controls ($n = 6$, $p < 0.05$). **f** Proportions of Lin⁻Sca1⁻c-kit⁺ progenitor cells in Lin⁻ BM cells in BADGE-treated and control mice ($n = 6$). **a–e** BADGE showed no effect on proliferation of hematopoietic cells in normal mice ($p > 0.05$). Data represent the mean \pm SD, ** $p < 0.05$



hematopoietic cells in BM sections of tail vertebrae at weeks 2 and 3 post-chemotherapy in BADGE-treated mice compared to controls, with a significant increase in the number of normal sinuses at weeks 3 (Fig. 5a). This effect was supported by BM cell density analysis. BM cell counts from the two groups reached normal levels by week 4 (Fig. 5c). To further investigate the hematopoietic capacity, total tail marrow was harvested and the number of BM cells was determined. The total number of BM cells was significantly higher in BADGE-treated mice compared to the controls (Fig. 5d). Analysis of these cells by flow cytometry revealed an increase in the proportion of CD45⁺ BM cells in the BADGE-treated mice compared to controls (Fig. 5e). BADGE induced no such effect on hematopoietic proliferation in normal healthy mice (Fig. 5b–e).

MPCs lost the ability of supporting Lin⁻ BM cell proliferation after differentiating into adipocytes in vitro

In the contact co-culture system, few BM cells detected in wells containing more adipocytes, while a significantly higher number of BM cells were observed in co-cultures treated with BADGE (Fig. 6a, c). The highest frequency of Lin⁻-derived BM cells was observed in MPC co-cultures where no adipocytes were present, serving as a positive control (Fig. 6b, c). It is known that MPCs act in a supportive role in hematopoiesis [25]. To date, treatment with BADGE has not been found to affect the proliferation of MPCs (Fig. 6d). Similarly, BADGE was also shown by Naveiras to have no direct effects on BM cells [11]. Taken

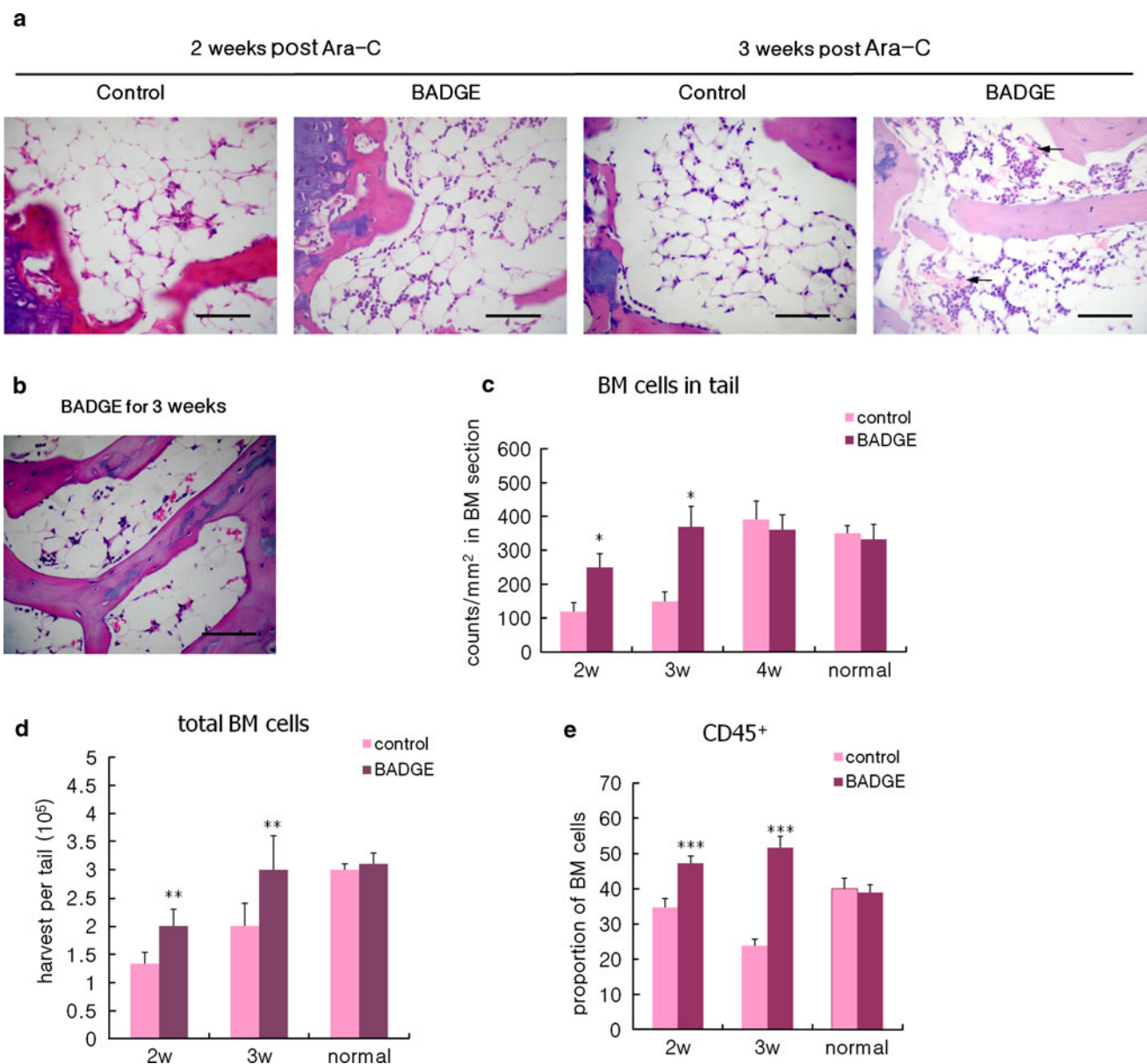


Fig. 5 **a** Increased infiltration of hematopoietic cells in BM sections of tail vertebrae from BADGE-treated mice following Ara-C administration, compared to control mice ($n = 6$, scale bar 100 μm , HE staining). **b** BM sections of tail from BADGE-treated mice without Ara-C treatment (HE staining, scale bar 100 μm). **c** Hematopoietic cells per mm^2 in tail vertebrae BM sections from BADGE-treated and control mice post Ara-C treatment ($n = 6$ in

weeks 2 and 3 groups, $n = 3$ in week 4 groups). **d** Number of total marrow cells obtained from tails of BADGE-treated and control mice ($n = 6$). **e** Proportions of CD45⁺ BM cells in tail BM of BADGE-treated and control mice ($n = 6$). **c–e** BADGE showed no effect on proliferation of hematopoietic cells in normal mice ($p > 0.05$). Black arrows sinuses. Data represent the mean \pm SD, * $p < 0.01$, ** $p < 0.05$, *** $p < 0.02$

together, these results demonstrate that adipocytes significantly inhibit the proliferation of Lin⁻ BM cells in vitro.

Adipocytes chemoattract Lin⁻ cells migration in a co-culture system in vitro

Since proliferation of hematopoietic cells was enhanced in BADGE-treated group during stress, we wondered what happened to the BM niche when fat cells hyperplasia.

To study the chemotactic effect of adipocytes on hematopoietic cells, we investigated the migration of Lin⁻ cells in the co-culture system containing the two kinds of cells. Interestingly, less Lin⁻ cells migrated in the BADGE-treated co-culture system (including less fat cells), compared to the control (including more fat cells) (Fig. 7a, c). The chemokine SDF-1 α was considered as a vital factor during the migration of hematopoietic cells [26]. As expected, SDF-1 α level was significant lower in the supernatant fluid in the BADGE-

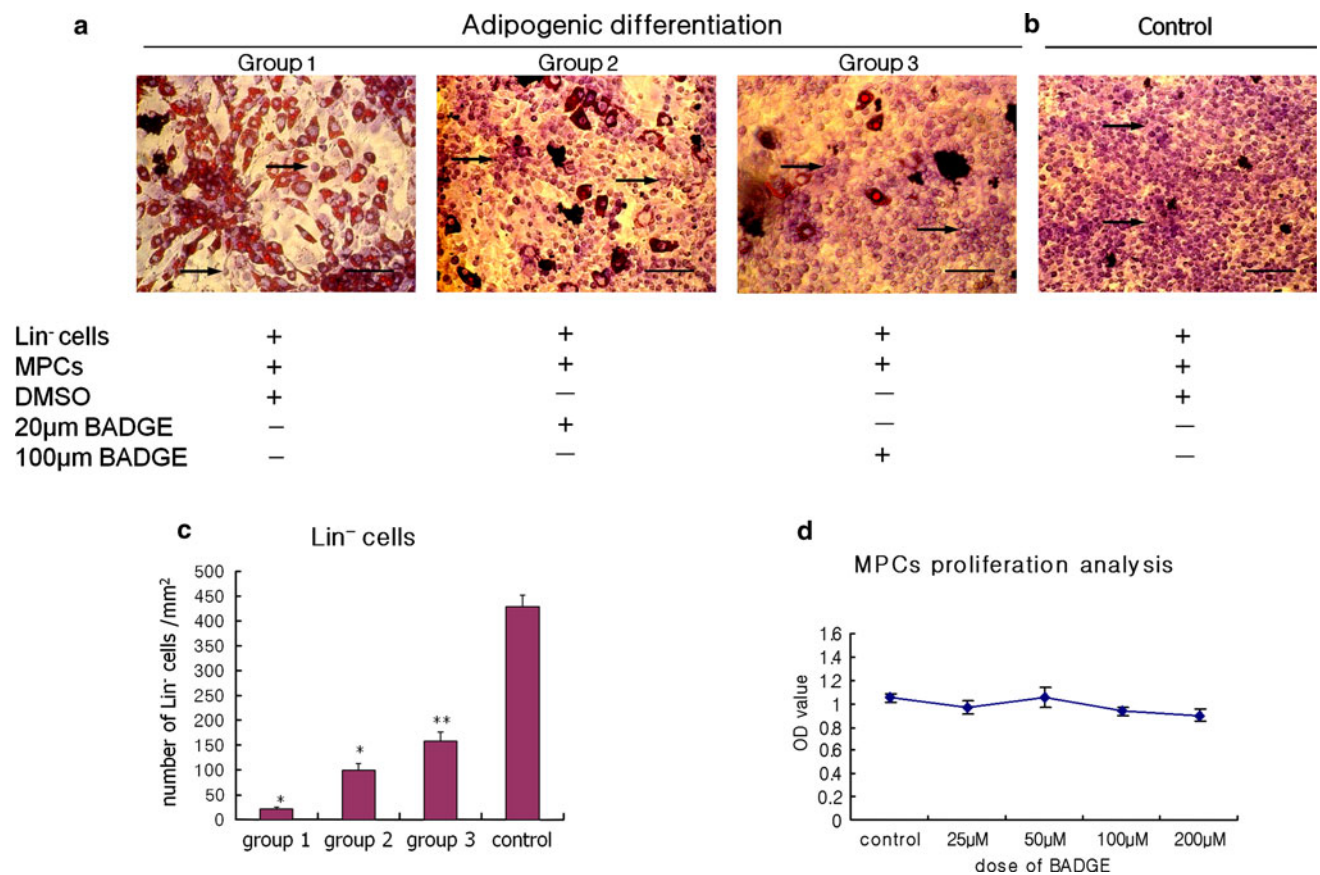


Fig. 6 **a** Lineage negative bone marrow (Lin⁻ BM) cells were co-cultured with BM stromal progenitor cells (MPCs) after adipogenic differentiation. The number of adipocytes derived from MPCs was controlled via treatment with different doses of BADGE (Oil red O staining for adipocytes and Wright's staining for Lin⁻ BM cells, scale bar 200 µm). **b** Lin⁻ BM cells were co-cultured with MPCs without adipocytes (Wright's staining for Lin⁻ BM cells, scale bar 200 µm).

c Less Lin⁻ cells in the co-culture system containing various number of adipocytes, compared to controls. **d** BADGE showed no effect on the proliferation of MPCs after treatment with increasing doses for 3 days ($p > 0.05$). Black arrows Lin⁻ BM cells and their derived cells. Data represent the mean of three independent wells \pm SD. * $p < 0.01$; ** $p < 0.05$

treated co-culture system, compared to the controls (Fig. 7d), though BADGE did not show effect on Lin⁻ cells migration when the Lin⁻ cells were cultured alone (Fig. 7b, c). To make sure the SDF-1 α level expressed individually during the adipogenic differentiation, SDF-1 α was evaluated when the pre-adipocyte cell line 3T3-L1 cells were differentiated into various number of fat cells. BADGE-treated 3T3-L1 cells expressed the least SDF-1 α mRNA level (Fig. 7e). In particular, the level of SDF-1 α expression was also lower in both of the long BM and tail BM in the BADGE-treated mice post-chemotherapy than in the controls (Fig. 7f, g).

HIF-1 α expression in BM decreases in vivo in BADGE-treated mice

It was well considered that the 'red marrow' contains more vascularity than the 'yellow marrow'. We supposed that blood flow and supply of oxygen may be different within the two niches. The transcription factor HIF-1 α is a key

signal in the cellular response to the hypoxia niche. Moreover, HIF-1 α controlled SDF-1 expression in the hypoxic niche [27]. Thus, we detected HIF-1 α expression to evaluate the level of hypoxia in BM cells in the adipocytes hyperplasia niche induced by chemotherapy. Moreover, on the basis of above findings, more LSK HSCs entered into cell cycle of proliferation. HSCs maintain cell cycle quiescence in G₀ phase of cycle through the precise regulation transcription factor HIF-1 α levels in their nucleus [28, 29]. Supposing its expression could be different in the two groups, mRNA level of *HIF-1 α* was detected by RT-PCR. *HIF-1 α* expression did not show much variation among those groups in the co-culture system in vitro (Fig. 8a). However, *HIF-1 α* level enhanced in long BM after Ara-C treatment, compared with the normal healthy mice (Fig. 8b). Interestingly, the BADGE-treated group showed significant expression lower than the control groups. The difference reached to the highest level on week 2 and declined along with time, with similar level on week

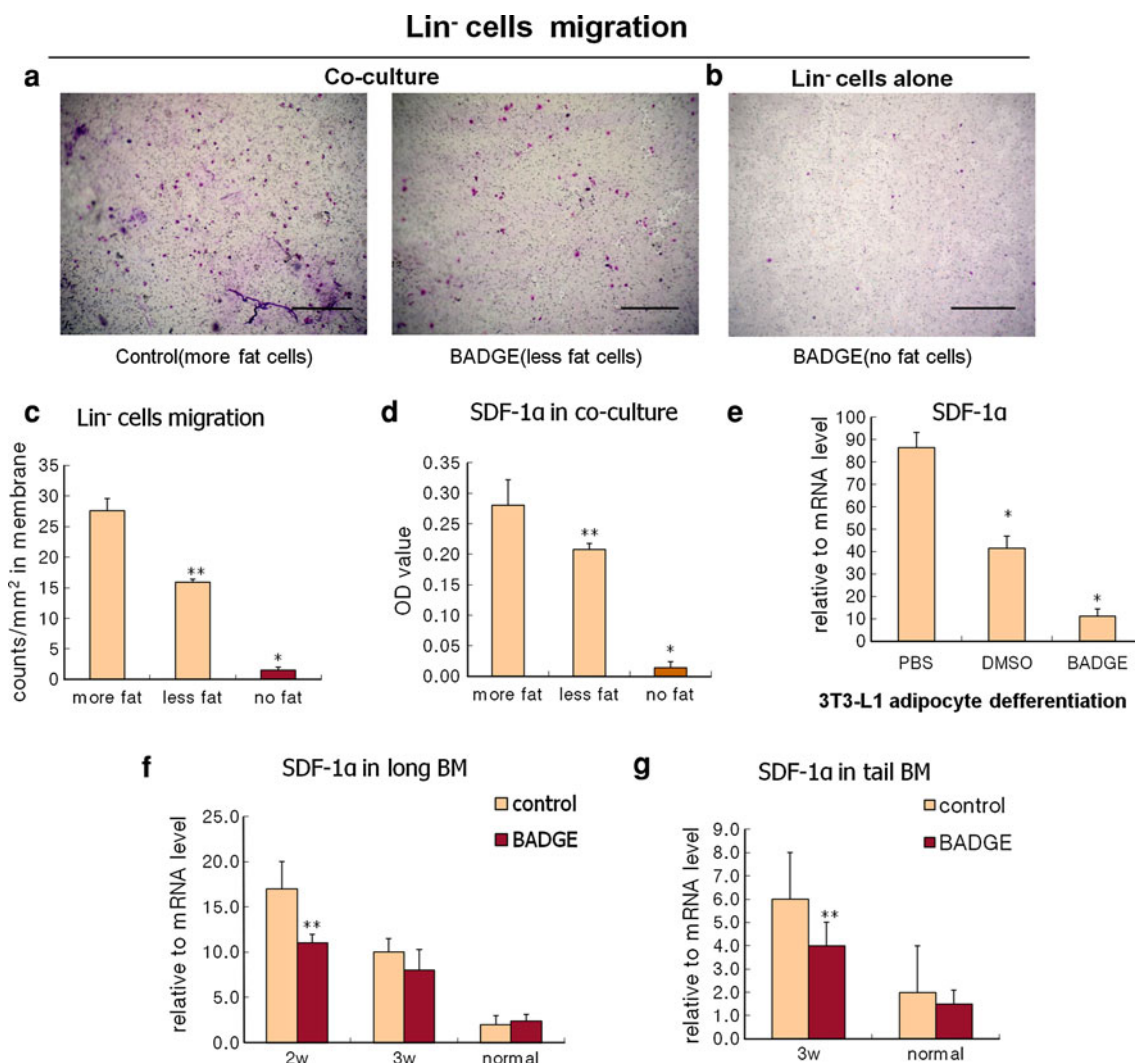


Fig. 7 a–c Lin⁻ cell migration capacity in different co-culture systems containing various adipocytes (Wright’s staining for Lin⁻ BM cells, scale bar 200 μm). **d** The SDF-1α levels in supernatant fluid were different co-culture system containing different number of adipocytes. **e** The SDF-1α expression was evaluated when the pro-

adipocyte line 3T3-L1 was adipogenic differentiated into different number of adipocytes. **f, g** The SDF-1α expression level was reduced in both long BM and tail BM of BADGE-treated mice ($n = 3$). Data represent the mean of three independent wells \pm SD. * $p < 0.01$; ** $p < 0.05$

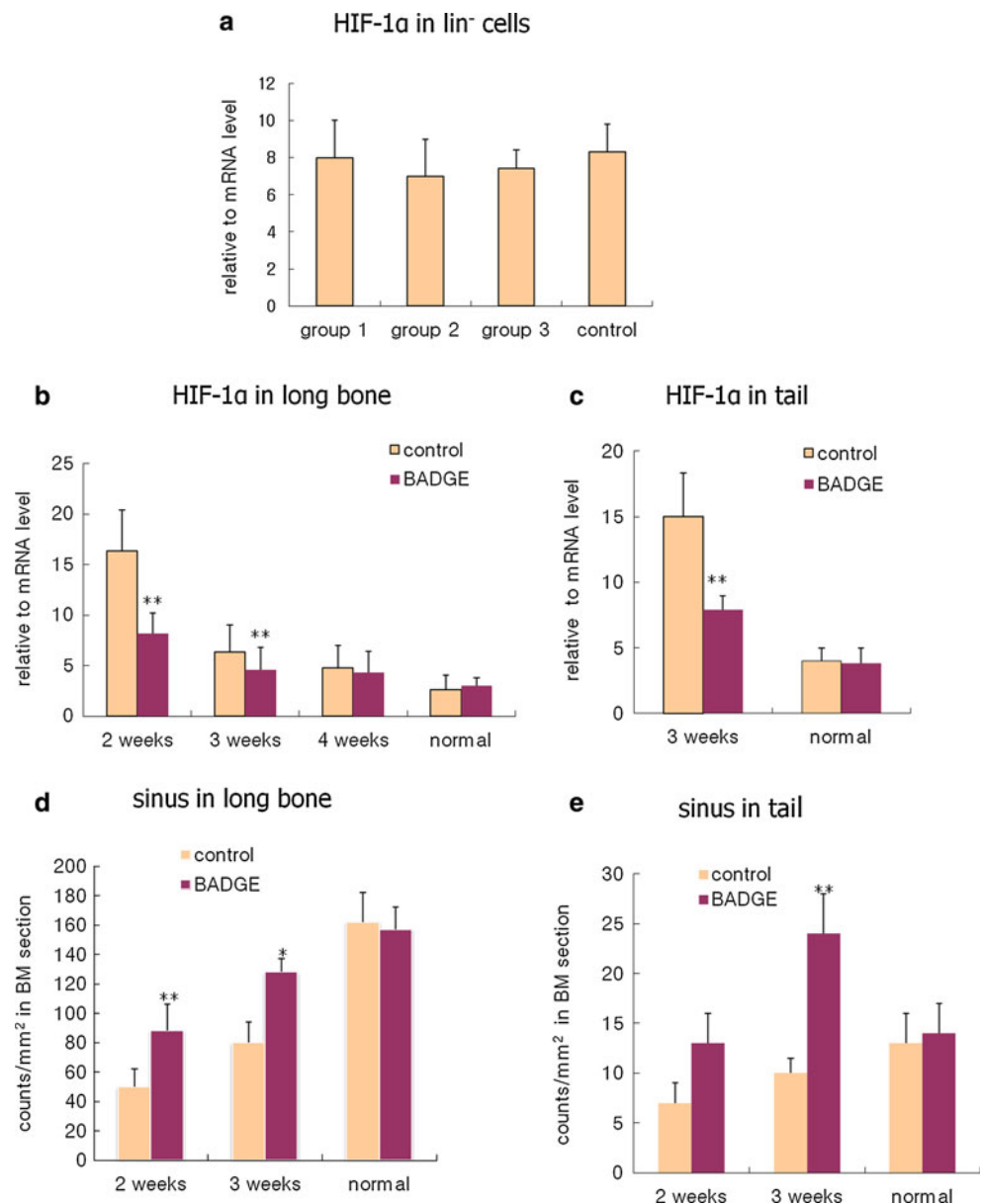
4, when hematopoiesis recovered (Fig. 8b). Similarly result was found on week 3 after chemotherapy in tail BM (Fig. 8c). In addition, as the component of oxygen supply in the BM niche, more sinuses regenerated in the BADGE-treated mice (Fig. 8d, e). BADGE did not promote an increase of sinuses in normal healthy mice (Fig. 8d, e). Whether this phenomenon could explain the decrease expression of *HIF-1α* needs further more research.

Discussion

Chemotherapy agents act as a ‘double edged sword’ in clinical leukemia research, killing malignant cells, but also

damaging the bone marrow niche [30]. Consistent with our study, hyperplastic adipocytes can be also induced by methotrexate (MTX) in rat marrow, and MTX promoted marrow stromal progenitor cells to form adipocytes in vitro [31]. This indicates that induction of adipocyte hyperplasia may not be unique to one chemotherapeutic drug. Our results also indicate that this is a transient and reversible process, often lasting 2 or 3 weeks. We found that the increase in adipocytes observed between weeks 1 and 3 declined as the hematopoietic system recovered. Similarly, adipocytes induced by MTX declined on day 14 [31]. Previous reports have shown that aplastic anemia can be induced by intensive and chronic chemotherapy [5, 6]. Whether this is also associated with irreversible

Fig. 8 **a** HIF-1 α expression showed no difference between these groups ($p > 0.05$). **b** HIF-1 α expression in long bone BM was lower in the BADGE-treated groups than the control groups, compared with normal mice treated with only BADGE ($n = 4$). **c** HIF-1 α expression of tail vertebra BM from BADGE-treated groups and control groups on week 3 after Ara-C administrated, compared to the controls and the normal mice ($n = 3$). **d** Number of sinuses increased in the long BM in BADGE-treated mice, compared to the controls and the normal mice ($n = 3$). **e** Number of sinuses increased in the long BM in BADGE-treated mice, compared to the controls and the normal mice ($n = 3$). * $p < 0.01$; ** $p < 0.05$



adipocyte hyperplasia in the BM may be the subject of future studies.

BADGE has been shown to prevent long bone marrow adipocyte formation after irradiation treatment in murine models [11]. In this study, we demonstrate for the first time that BADGE is also an effective inhibitor of adipogenesis in both long bone marrow (red marrow) and the tail marrow (yellow marrow), following chemotherapy in vivo.

Up to now, few studies have investigated the role of fat cells in hematopoietic recovery following chemotherapy. We observed a higher number of peripheral WBCs and neutrophils in BADGE-treated mice, and a trend toward increased platelets. While we did not observe significant differences in hemoglobin levels in BADGE and control-treated mice, this may be due to the longer life span of red

blood cells. Further studies are required to understand the influence of adipocytes on the growth of hematopoietic progenitors. In this study, we also observed that the effect of BADGE treatment was lost upon hematopoietic recovery, and this agent did not effect on normal mice under homeostasis. In addition, we also confirmed previous studies that this agent displays no direct effect on the expansion of hematopoietic progenitors [11].

These data suggest that the effect of BADGE on hematopoiesis occurs only under hematopoietic stress, for example following treatment with chemotherapeutic drugs, which coincides with a transient increase in adipocytes, thus inhibiting hematopoietic recovery. Thus, treatment with PPAR γ inhibitor indirectly affects hematopoietic recovery. This differs from the mechanism underlying

treatment with G-CSF, which induces hematopoietic expansion directly, both under homeostasis and stress [32]. High expression of *Nrp1* has previously been also detected in adipocytes, and was shown to inhibit the production of G-CSF by macrophages [33]. In this study, we showed that expression of *Nrp1* was also decreased in adipocyte-inhibited mice. However, the G-CSF secretion might not be the mechanism of hematopoietic recovery in adipocyte-inhibited group according to our study. By contrast, BADGE exerted its effect on hematopoietic recovery corresponding with dynamic number of adipocytes in BM.

We observed an improvement in hematopoiesis in the long BM of adipocyte-inhibited mice. As previously reported, HSCs reside predominantly in the G₀ fraction and remain in a resting status [34]. Moreover, it was reported that there was a decrease in the number of HPCs and a higher percentage of quiescent CD34⁺ HSCs in adipocyte-rich BM in mice [11]. Thus, we conclude that inhibition of adipogenesis stimulates the proliferation of hematopoietic progenitors and LSK HSCs. Since we observed relatively less proliferative LSKs in the control group, we hypothesize that the presence of adipocytes in the BM may retain more HSCs in the G₀ phase of the cell cycle. In addition, since we did not observe a significant increase in the proportion of LSK HSCs, in keeping with the observed increase in HPCs, we hypothesize that differentiation of LSK HSCs is promoted in the adipocyte-inhibited mice, although this should be investigated in greater detail in the future. These data demonstrate that the formation of adipocytes may negatively affect hematopoietic recovery following Ara-C treatment in the long bone marrow.

The BM of adult tail vertebrae are filled with fat cells and hypoplastic hematopoietic cells, indicating ‘fatty marrow’. Under stress conditions, however, this is converted into ‘red marrow’ with increased hematopoietic capacity [35, 36]. In this study, we did not observe an increase in BM cells in the tail vertebrae following Ara-C treatment; however, this may reflect that the low intensity of stress induced by Ara-C was not high enough to induce compensatory hematopoiesis in the fatty marrow. We did observe a significant increase in hematopoietic cells in the BM of tail vertebrae at weeks 2 and 3 after Ara-C treatment in BADGE-treated mice compared to controls, with no further increase in BM cells at week 4. This result is consistent with the decreased number of adipocytes observed in tail BM at weeks 2 and 3 in BADGE-treated mice. Whether this increase in tail BM hematopoietic cells is due to migration from the ‘red marrow’ of long bones or proliferation in situ remains unclear. This finding may shed light on how to convert the ‘fatty marrow’ niche to ‘active marrow’ in specific hematopoietic hypoplasia disorders.

Since the exact mechanism underlying this phenomenon has not yet been defined, we study the primary mechanism

about changes in the BM niche affected by the adipocytes in this research.

First, adipocytes may affect hematopoietic cells proliferation and migration through a direct way.

1. In the co-culture system in our study, BM stromal progenitors lost their ability to support BM cell proliferation after differentiating into mature adipocytes in vitro. Furthermore, the cytokine SCF, which support hematopoietic cell growth [37], were down-regulated in adipocytes. So we infer SCF would be one of the hematopoietic factors that act the effect.
2. HPCs migrate in vitro and in vivo toward a gradient of the chemokine SDF-1 produced by stromal cells [26, 38]. In this study, adipocytes chemoattracted Lin⁺ cells migration in a co-culture system in vitro, secreting a higher level of SDF-1 α in the co-culture system. Furthermore, SDF-1 could play multiple roles on hematopoietic progenitors besides chemoattraction, such as survival and cell cycling [39]. The BM niche including rich adipocytes contains more primary HSCs in it [11]. Thus, this result may reflect a particular niche near the hyperplasia adipocytes in the BM. However, unlike the co-culture study in vitro, there were other components in the BM niche in mice that also produced SDF-1 α , such as the osteoblasts and the endothelial [40–42]. Thus, more studies in situ are needed to indicate the precise effect of adipocyte on hematopoietic cells in vivo.

Second, adipocytes also impacted the hematopoietic proliferation ability in an indirect way.

Hypoxic niche did an important role to regulate the proliferation of hematopoietic cells. Hyperplastic fat cells acted as a barrier to block the hematopoietic cells to reside in the oxic niche near the sinuses. Adipocytes were observed around or attached to the pathological sinuses following Ara-C treatment in our study. This finding supports the recent report that adipocyte progenitors reside in the mural cell compartment of the adipose vasculature or near endothelial cells [43, 44]. Thus, a more hypoxic niche should exist in the adipocyte hyperplastic BM. We supposed that blood flow and supply of oxygen may be different within the two niches. As an important key signal in the cellular response to the hypoxic niche [45–48], HIF-1 α was proved to be a lower level in the adipocyte-inhibited mice.

Hyperplastic adipocytes may interact with disrupted sinus endothelial cells under stress. In this study, echoing the decreased expression of HIF-1 α , an increase number of sinuses were detected in adipocytes-inhibited mice (Figs. 2a, 5a, 8d, e). A recent report showed that injection of endothelial cells into the BM of irradiated mice promoted regeneration of vessels and elimination of

adipocytes [49]. In another report describing the effects of adipocytes on vascularity, adiponectin induced apoptosis of endothelial cells via caspase signaling, and significantly inhibited VEGF-induced migration of endothelial cells [50]. It was well considered that the ‘red marrow’ (few fat cells included) contains more vascularity than the ‘yellow marrow’ (more fat cells included). We therefore suggest that hyperplastic adipocytes may interact with disrupted sinus endothelial cells under stress, which suggested a plausible mechanism for impacting the BM niche in an indirect way by adipocytes.

It was reported that HIF-1 induced SDF-1 expression under hypoxic condition to regulate progenitor cells trafficking [27]. A decreased HIF-1 α expression and SDF-1 α expression were both found in the BADGE-treated mice in this our study, corresponding to the previous study.

Our study sheds a unique light on the effects of adipocytes on hematopoiesis, post stress. During the course of clinical treatment, patients with leukemia and those preparing for bone marrow transplantation are typically treated with intensive and high-dose chemotherapy. This process may result in bone marrow suppression in conjunction with adipocyte hyperplasia. Inhibition of adipogenesis following chemotherapy may represent a new method to preserve the BM niche and improve hematopoietic recovery. Thus, the hematopoietic niche can be converted from a ‘fertile niche’ to a ‘barren niche’ through controlling the number of adipocytes present. However, several questions still remain. Why and how are adipocytes generated in the BM under conditions of stress? How do adipocytes interact with sinus endothelial cells? Do adipocytes effect on hematopoietic relative factors in BM in vivo? Do PPAR γ inhibitors affect other components in marrow niche at the same time? More research is required to adequately resolve these questions.

In summary, we demonstrate that adipogenesis may be induced by chemotherapeutic agents, and prevented by treatment with the PPAR γ inhibitor, BADGE. Hematopoietic recovery post-chemotherapy may be improved in adipocyte-inhibited mice, not only in the active marrow, but also in the fatty marrow. This study provides evidence that adipocytes may disturb the BM niche and hematopoietic recovery after chemotherapy. To explore the mechanism underlying this phenomenon, further studies focusing on the effect of adipocytes on sinus endothelial cells as well as hematopoietic relative factors are necessary.

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Conflict of interest The authors indicate no potential conflict of interest.

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