Published online: April 27, 2015

Article

SOURCE DATA | TRANSPARENT PROCESS

THE EMBO JOURNAL

Neuropeptide Y regulates the hematopoietic stem cell microenvironment and prevents nerve injury in the bone marrow

Min Hee Park[1,2,3,†], Hee Kyung Jin[1,4,†], Woo-Kie Min[5], Won Woo Lee[6], Jeong Eun Lee[7], Haruhiko Akiyama[8], Herbert Herzog[9], Grigori N Enikolopov[10], Edward H Schuchman[11] & Jae-sung Bae[1,2,3,*]

Abstract

Many reports have revealed the importance of the sympathetic nervous system (SNS) in the control of the bone marrow environment. However, the specific role of neuropeptide Y (NPY) in this process has not been systematically studied. Here we show that NPY-deficient mice have significantly reduced hematopoietic stem cell (HSC) numbers and impaired regeneration in bone marrow due to apoptotic destruction of SNS fibers and/or endothelial cells. Furthermore, pharmacological elevation of NPY prevented bone marrow impairments in a mouse model of chemotherapy-induced SNS injury, while NPY injection into conditional knockout mice lacking the Y1 receptor in macrophages did not relieve bone marrow dysfunction. These results indicate that NPY promotes neuroprotection and restores bone marrow dysfunction from chemotherapy-induced SNS injury through the Y1 receptor in macrophages. They also reveal a new role of NPY as a regulator of the bone marrow microenvironment and highlight the potential therapeutic value of this neuropeptide.

Keywords bone marrow microenvironment; hematopoietic stem cell; neuropeptide Y; regeneration; sympathetic nervous system
Subject Categories Neuroscience; Signal Transduction; Stem Cells
DOI 10.15252/embj.201490174 | Received 26 September 2014 | Revised 30 March 2015 | Accepted 1 April 2015 | Published online 27 April 2015
The EMBO Journal (2015) 34: 1648–1660

Introduction

The bone marrow endosteal and perivascular microenvironments support the function of hematopoietic stem cells (HSCs) (Ding *et al*, 2012). These bone marrow microenvironments consist of functional compartments that regulate HSC retention, survival, self-renewal, and differentiation (Arai *et al*, 2004; Scadden, 2006; Sugiyama *et al*, 2006; Butler *et al*, 2010; Lévesque *et al*, 2010). Endothelial cells (ECs) and nestin$^+$ mesenchymal stem cells (MSCs) found in the perivascular microenvironment regulate HSCs survival and bone marrow regeneration (Butler *et al*, 2010; Méndez-Ferrer *et al*, 2010; Ehninger & Trumpp, 2011; Doan *et al*, 2013), and the sympathetic nervous system (SNS) and macrophages are positive regulators of ECs and nestin$^+$ MSCs, which retain HSCs (Winkler *et al*, 2010; Chow *et al*, 2011).

Recent studies have reported that bone marrow innervations regulate HSC microenvironments. Sympathetic nerve fibers synapsed on perivascular cells are associated with HSC hibernation in bone marrow (BM). Moreover, bone marrow nerve injury induces impairment of HSC regeneration (Méndez-Ferrer *et al*, 2008; Yamazaki *et al*, 2011; Lucas *et al*, 2013). Neurotransmitters released from the SNS also play a key regulatory role in bone marrow microenvironments. In particular, release of acute physiological stress-induced neurotransmitters such as substance P, dopamine, and norepinephrine regulate regeneration, motility, and proliferation of bone marrow cells (Katayama *et al*, 2006; Spiegel *et al*, 2007; Hong *et al*, 2009).

Neuropeptide Y (NPY) is a neurotransmitter that is often released by sympathetic nerves in the central or peripheral nervous system, and also functions as a orexigenic peptide inducing food intake

1 Stem Cell Neuroplasticity Research Group, Kyungpook National University, Daegu, Korea
2 Department of Physiology, Cell and Matrix Research Institute, School of Medicine, Kyungpook National University, Daegu, Korea
3 Department of Biomedical Science, BK21 Plus KNU Biomedical Convergence Program, Kyungpook National University, Daegu, Korea
4 Department of Laboratory Animal Medicine, College of Veterinary Medicine, Kyungpook National University, Daegu, Korea
5 Department of Orthopaedic Surgery, Kyungpook National University Hospital, Daegu, Korea
6 Department of Microbiology and Immunology, Seoul National University College of Medicine, Seoul, Korea
7 Department of Radiation Oncology, Kyungpook National University Hospital, Daegu, Korea
8 Department of Orthopaedics, Kyoto University, Kyoto, Japan
9 Neuroscience Research Program, Garvan Institute of Medical Research, Sydney, NSW, Australia
10 Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, NY, USA
11 Department of Genetics and Genomic Sciences, Icahn School of Medicine at Mount Sinai, New York, NY, USA
*Corresponding author. Tel: +82 53 420 4815; Fax: +82 53 424 3349; E-mail: jsbae@knu.ac.kr
†These authors contributed equally to this work

(Zukowska-Grojec, 1995; Kalra & Kalra, 2004; Kuo *et al*, 2007a). Previous studies have demonstrated that NPY regulates immune cell homeostasis, bone homeostasis, or vascular remodeling through Y receptors expressed in bone marrow cells, especially macrophages, osteoblasts, and ECs (Wheway *et al*, 2005; Kuo *et al*, 2007b; Lee & Herzog, 2009). Despite these associations between NPY and bone marrow cells, the specific role of NPY in the HSC microenvironment has not been fully characterized.

Here we show that NPY deficiency causes impairment of HSC survival and bone marrow regeneration, as well as integrity of SNS fibers and/or ECs, and that pharmacological NPY increase leads to HSC regeneration through the Y1 receptor, suggesting a new role of NPY in the bone marrow microenvironment. Furthermore, NPY treatment prevents cisplatin-induced deficits in bone marrow function through the Y1 receptor in macrophages, indicating the potential for regulating NPY activity as a new therapeutic intervention for chemotherapy-induced sensory neuropathy or bone marrow dysfunction.

Results

NPY deficiency in the HSC microenvironment

We first confirmed the lack of NPY expression in BM of NPY knockout ($NPY^{-/-}$) mice (Fig 1A) and then analyzed the bone marrow cell composition in wild-type (WT) and $NPY^{-/-}$ mice. Although no significant difference in the numbers of bone marrow nucleated cells (BMNCs) was observed between WT and $NPY^{-/-}$ mice, the percentage of phenotypic $Lin^-Sca1^+c\text{-}Kit^+$ (LSK) cells, $LSKCD48^-CD150^+$ long-term HSCs (LT-HSCs), and $CD45^-Lin^-CD31^-Sca1^+CD51^+$ MSCs was significantly reduced in $NPY^{-/-}$ mice (Fig 1B–D). The numbers of hematopoietic stem/progenitor cells (HSPCs) in peripheral blood (PB) also were significantly decreased (Fig 1E). To confirm whether reduced numbers of HSPCs in the PB were derived from their reduced numbers in the BM, we performed migration assays. The results showed no significant difference between migrated LSK cells of WT and $NPY^{-/-}$ mice with or without SDF-1α (Fig 1F). These results indicated that NPY-deficient HSPCs had normal motility and migration capacity and that reduction of HSPCs in the PB of $NPY^{-/-}$ mice was likely derived from their reduced numbers in the BM. We next investigated whether the reduced HSC numbers in the BM of $NPY^{-/-}$ mice was a cell-autonomous effect *in vivo* and *in vitro*. LSK cells of WT and $NPY^{-/-}$ mice were stained with annexin V, resulting in no difference of apoptosis between the two groups (Fig 1G). To further confirm apoptosis *in vitro*, LSK cells sorted from the BM of WT mice were treated with NPY siRNA for 48 h, and the effects on reduced NPY expression were confirmed (Fig 1H). Apoptosis and expression of pro- or anti-apoptotic genes were not changed between the two groups (mice treated with control or NPY siRNA) (Fig 1I and J). Therefore, the reduction of HSCs in $NPY^{-/-}$ mice was not due to cell-autonomous apoptosis. Cell cycle analysis using an antibody to Ki-67 showed that 20% more LSK cells were quiescent (in the G0 phase) in $NPY^{-/-}$ mice. However, the S-G2-M phase did not change (Fig 1K). Of note, NPY deficiency did not alter the number of macrophage subsets, B cells, T cells, and complete blood counts (Supplementary Fig S1A–D and Supplementary Table S1). Moreover, NPY deficiency did not affect survival (Fig 1D) or apoptosis (Supplementary Fig S1E–G) of osteoblasts differentiated from MC3T3-E1 pre-osteoblasts. These results suggested that NPY deficiency led to the impairment of HSC survival, but did not affect their differentiation in the bone marrow microenvironment.

To investigate further the contribution of NPY in the mediation of HSC engraftment and repopulating efficiency in the marrow microenvironment, we transplanted WT $CD45.1^+$ BMNCs into lethally irradiated WT or $NPY^{-/-}$ mice. Transplantation into $NPY^{-/-}$ mice resulted in significantly reduced mouse survival compared to WT mice, and bone marrow regeneration was severely reduced as well (Fig 2A and B). LSK cells also were significantly decreased in the BM of $NPY^{-/-}$ mice after transplantation (Fig 2C). Homing to the BM and migration to the appropriate microenvironment are required for engraftment of transplanted HSCs (Lapidot *et al*, 2005). We therefore performed a homing assay in transplanted WT and $NPY^{-/-}$ mice (Fig 2D). The result showed that NPY did not affect the homing of hematopoietic progenitors to the BM (Fig 2E and F), suggesting that the reduced HSC survival in $NPY^{-/-}$ mice is independent of homing. To further confirm HSC repopulating activity and the role of NPY in this phenotype, we injected 5-fluorouracil (5FU), which causes ablation of most hematopoietic cells, into WT or $NPY^{-/-}$ mice to induce repopulation from remaining HSCs. $NPY^{-/-}$ mice showed marked reductions in mouse survival, the number of BMNCs and complete blood counts, and percentage of HSCs at day 12 after 5FU injection (Fig 2G–I and Supplementary Table S1). Overall, these results indicated that NPY was required for HSC survival and bone marrow regeneration.

Next, we transplanted WT or $NPY^{-/-}$ BMNCs into lethally irradiated WT mice to determine the effect of NPY deficiency in BMNCs on bone marrow regeneration. The results showed no significant difference in mouse survival, repopulating efficiency, and the

Figure 1. NPY deficiency induces impairment of HSC survival in BM.

A NPY mRNA expression relative to Gapdh (n = 6 mice per group) in the BM of WT or $NPY^{-/-}$ mice.
B, C Number of BMNCs (B) and percentage of LSK cells or LT-HSCs (C) in BM of WT (n = 6–9) or $NPY^{-/-}$ mice (n = 6–10).
D The percentage of $CD45^-Lin^-CD31^-Sca1^+CD51^+$ MSCs and $CD45^-Lin^-CD31^-Sca1^-CD51^+$ osteoblasts in BM of WT or $NPY^{-/-}$ mice (n = 4–6 mice per group).
E Number of colony-forming unit assay (CFU-C) in PB of WT or $NPY^{-/-}$ mice (n = 6 mice per group).
F Left, experimental scheme of the transwell migration assay. Right, comparison of the chemotactic ability of WT and NPY-deficient LSKs (n = 3).
G Percentage of apoptotic cells in LSK cells purified from the BM of WT or $NPY^{-/-}$ mice (n = 4 mice per group).
H Expression levels of NPY in LSK cells exposed to control or NPY siRNA (n = 3).
I Percentage of apoptotic cells in control or NPY siRNA-treated LSK cells (n = 3 mice group).
J Quantitative real-time PCR analysis for pro- or anti-apoptotic gene expression in each group (n = 3).
K Cell cycle analysis of LSK cells purified from the BM of WT or $NPY^{-/-}$ mice (n = 5 mice per group).

Data information: $^*P < 0.05$. All error bars indicate s.e.m.

Published online: April 27, 2015
The EMBO Journal

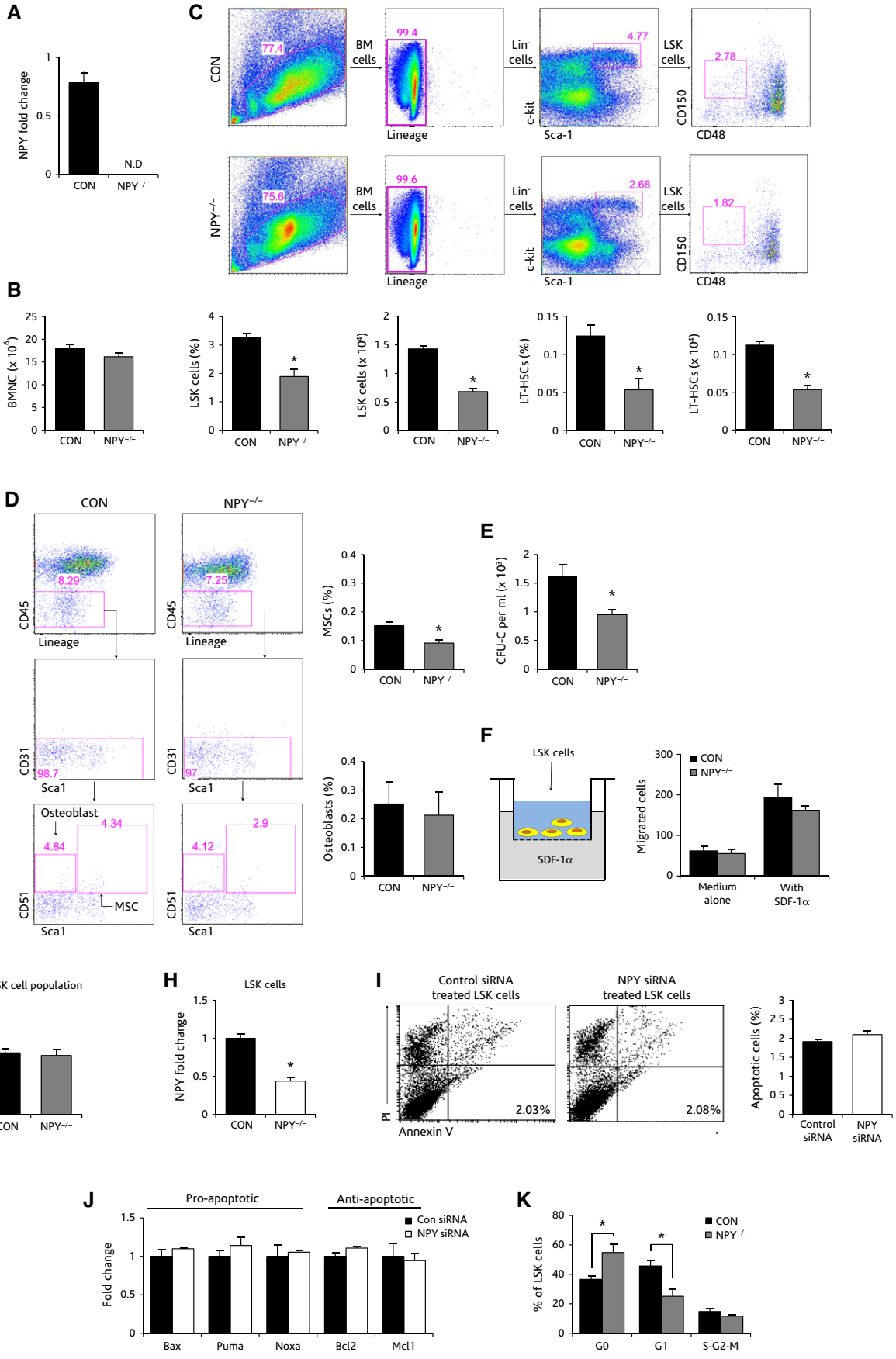

Figure 1.

Published online: April 27, 2015

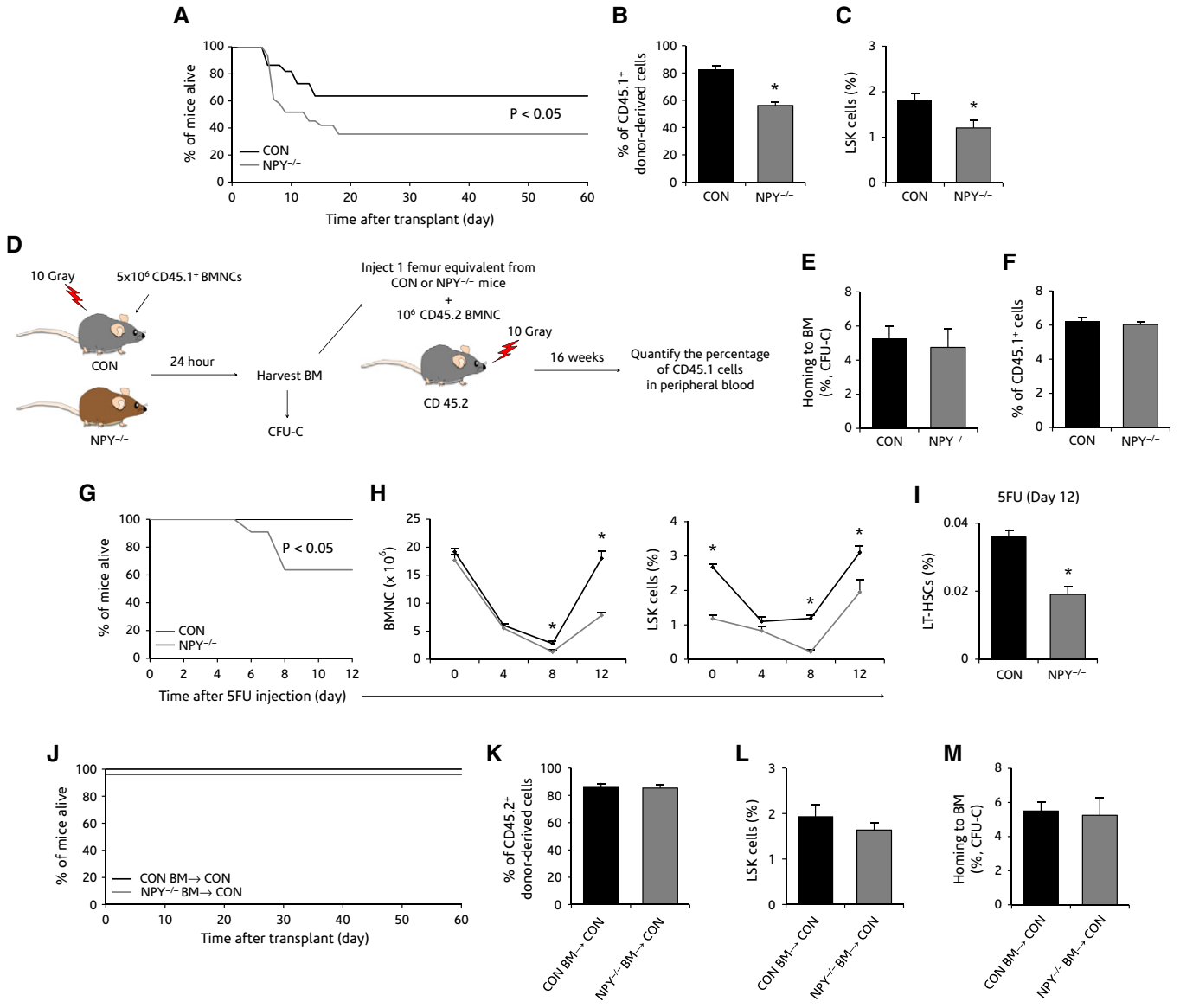

Figure 2. NPY is required for bone marrow regeneration.

A Survival of WT (n = 22) or NPY$^{-/-}$ (n = 31) mice after transplantation.

B, C Competitive repopulation assay showing the percentage of CD45.1$^+$ cells (B) in blood and the percentage of LSK cells (C) in BM 8 weeks after transplant in WT (n = 4–5) or NPY$^{-/-}$ mice (n = 4–5).

D Experimental design to determine the efficiency of HSC homing to the BM of WT or NPY$^{-/-}$ mice.

E Percentage of donor CFU-C detected in the BM of WT (n = 4) or NPY$^{-/-}$ mice (n = 4) at 24 h after lethal irradiation and injection of 5 × 10^6 donor WT BMNCs.

F Percentage of CD45.1$^+$ cells in PB of CD45.2$^+$ recipient mice 16 weeks after competitive transplant as depicted in (D) (n = 4 mice per group).

G Survival of NPY$^{-/-}$ (n = 11) mice compared to WT (n = 15) after administration of 5FU (i.v.).

H Number of BMNCs and percentage of LSK cells in BM of WT or NPY$^{-/-}$ mice at days 0, 4, 8, and 12 after 5FU injection (n = 4–6 mice per group).

I Percentage of LT-HSCs were determined at day 12 after 5FU injection (n = 4–6 mice per group).

J Survival of WT mice after WT or NPY$^{-/-}$ BMNCs transplantation (n = 15 per group).

K, L Competitive repopulation assay showing the percentage of (K) CD45.2$^+$ cells in blood and the percentage of (L) LSK cells in BM 8 weeks after transplantation (n = 5 per group).

M Percentage of donor CFU-C detected in the BM of 5 × 10^6 donor WT or NPY$^{-/-}$ BMNCs transplanted WT mice at 24 h after lethal irradiation (n = 4 per group).

Data information: *P < 0.05. All error bars indicate s.e.m.

percentage of LSK cells in BM of each group after transplantation (Fig 2J–L). Moreover, NPY$^{-/-}$ BMNCs had a normal homing ability to the BM (Fig 2M). These results indicated that NPY deficiency in bone marrow cells did not affect bone marrow regeneration.

Destruction of SNS fibers and/or ECs by NPY absence

Sympathetic nerves are required for HSC survival and bone marrow regeneration (Katayama *et al*, 2006; Méndez-Ferrer *et al*,

Published online: April 27, 2015
The EMBO Journal

2008; Lucas *et al*, 2013). Previous studies reported that SNS injury impairs hematopoietic function by reducing nestin$^+$ cells and ECs, and autonomic nervous system dysfunction affected the endothelial dysfunction (Lucas *et al*, 2013; Amiya *et al*, 2014). We therefore hypothesized that NPY$^{-/-}$ mice have defective SNS fibers in BM since NPY is a sympathetic neurotransmitter in the autonomic system. As expected, the results showed reduction of bone marrow SNS fibers in NPY$^{-/-}$ mice by staining with an antibody to the catecholaminergic enzyme tyrosine hydroxylase (Th) (Fig 3A). Moreover, the BM of NPY$^{-/-}$ mice contained fewer CD31$^+$ ECs compared to WT mice (Fig 3B). Taken together, these findings revealed that NPY deficiency caused impairment of HSC

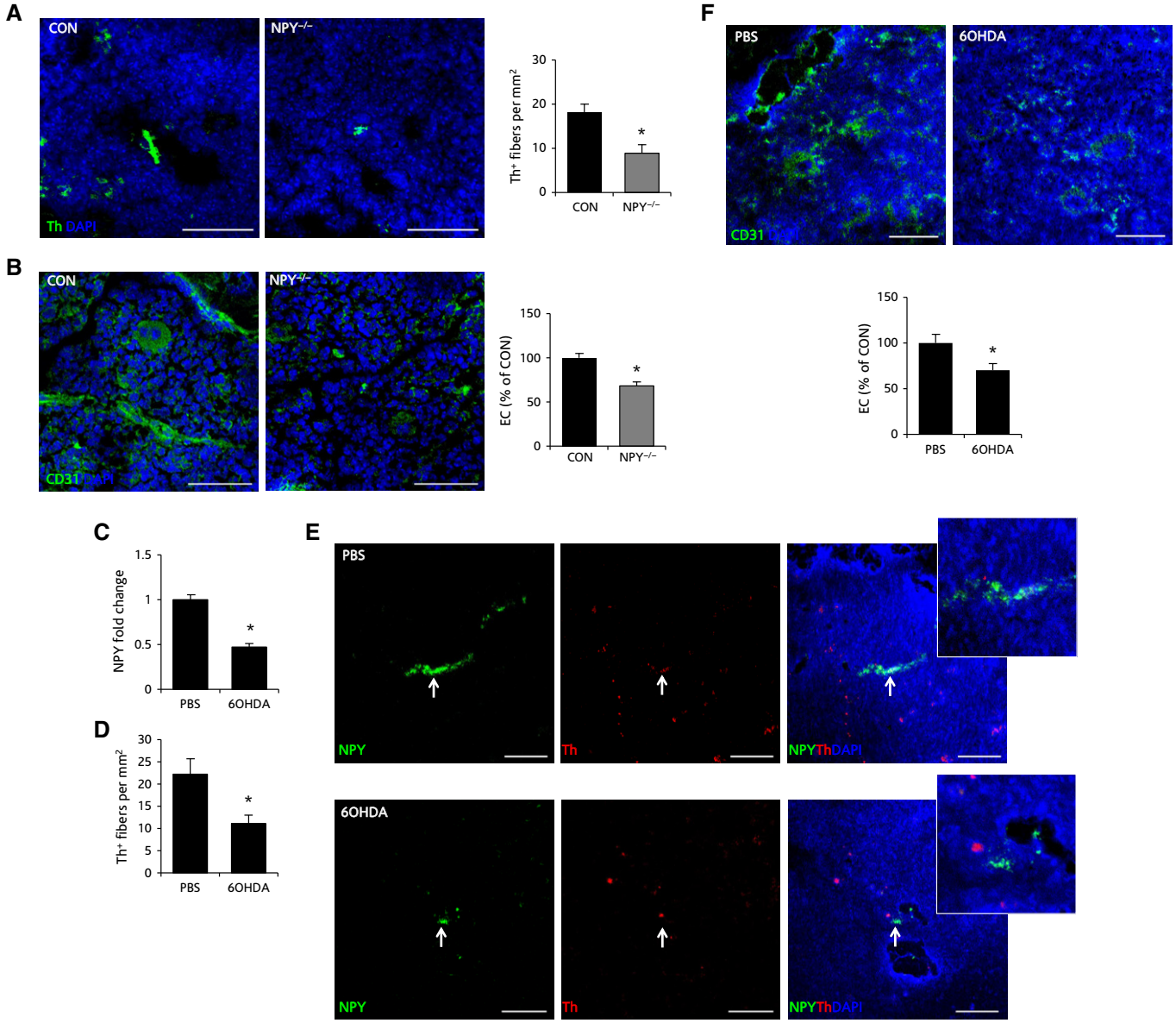

Figure 3. NPY deficiency causes the reduction of SNS fibers and ECs.

A Left, representative immunofluorescence images to detect the presence of Th$^+$ fibers. Scale bar, 50 μm. Right, quantification of Th$^+$ fibers in the BM of WT or NPY$^{-/-}$ mice (n = 6 mice per group).

B Left, representative immunofluorescence BM images of CD31$^+$ ECs. Scale bar, 40 μm. Right, number of CD31$^+$ ECs per femur in WT or NPY$^{-/-}$ mice (n = 6 mice per group).

C Expression of NPY in BM of PBS- or 6OHDA-treated mice (n = 6 mice per group).

D Quantification of Th$^+$ fibers in the BM of PBS- or 6OHDA-treated mice (n = 4 mice per group).

E Representative immunofluorescence images of BM showing NPY (green) merged with sympathetic neuron (Th, red). Scale bar, 40 μm.

F Representative immunofluorescence BM images of CD31$^+$ ECs and percentage of CD31$^+$ ECs per femur in PBS- or 6OHDA-treated mice (n = 4 mice per group). Scale bar, 30 μm.

Data information: *$P < 0.05$. All error bars indicate s.e.m.

survival and bone marrow regeneration by reducing the SNS fibers and/or CD31$^+$ ECs. To further reveal cause and effect relationships between SNS fibers and decreased numbers of ECs, as well as the expression of NPY, WT mice were injected with 6OHDA, which induces SNS fibers defects. 6OHDA-treated mice showed reduction of NPY expression and Th$^+$ SNS fibers (Fig 3C and D); low expression of NPY co-localized with Th$^+$ SNS fibers in BM (Fig 3E). The numbers of ECs also were decreased in the BM of 6OHDA-treated mice (Fig 3F). Therefore, these results indicated that destruction of SNS fibers affected ECs survival as well as NPY expression.

Previous studies reported that the p53 tumor suppressor gene in sympathetic neurons affects neuronal survival and that NPY is involved in the cell death mechanism (Trimmer *et al*, 1996; Corvino *et al*, 2012; Gonçalves *et al*, 2012). We first confirmed apoptosis levels in BM of WT or NPY$^{-/-}$ mice and found increased apoptosis in NPY$^{-/-}$ mice (Fig 4A). To gain more specific insights, we measured expression of p53 and several pro- or anti-apoptotic genes, which are the major checkpoints for the cell death pathway, and found increased p53 signaling and expression of pro-apoptotic genes in BM of NPY$^{-/-}$ mice (Fig 4B and C). Furthermore, apoptotic Th$^+$ cells or CD31$^+$ ECs *in vivo* were increased in the BM of NPY$^{-/-}$ mice (Fig 4D). NPY siRNA-exposed neurons or BM ECs *in vitro* showed reduction of NPY expression, also leading to increased apoptosis and p53 signaling, or expression of pro-apoptotic genes (Fig 4E–H). These findings indicated that NPY deficiency caused p53-dependent apoptosis of SNS fibers and/or CD31$^+$ ECs in the BM, resulting in impairment of HSC survival.

NPY/Y1 regulation in bone marrow microenvironment cell survival

We next investigated whether NPY or the Y1 receptor was involved in survival of SNS fibers and marrow microenvironment cells. To establish the appropriate NPY or Y1 agonist injection time, we first confirmed age-dependent changes of SNS fiber expression in BM of WT or NPY$^{-/-}$ mice and found significant reduction of Th$^+$ nerves in BM of 4-week-old NPY$^{-/-}$ mice (Fig 5A). Therefore, 2-week-old WT or NPY$^{-/-}$ mice were treated with NPY or a Y1 agonist ([Leu31, Pro34]NPY) intraperitoneally (i.p.) for 4 weeks. NPY treatment into NPY$^{-/-}$ mice significantly prevented loss of LSK cells and LT-HSCs in the BM of these mice (Fig 5B). The density of Th$^+$ fibers and the number of CD31$^+$ ECs in BM of NPY$^{-/-}$ mice also was increased by NPY treatment (Fig 5C and D). Similar effects were observed in Y1 agonist-injected NPY$^{-/-}$ mice (Fig 5E–G). Moreover, increased apoptosis in BM of NPY$^{-/-}$ mice was decreased with NPY or Y1 agonist treatment (Fig 5H). These data indicated that NPY/Y1 receptor regulation affected HSC survival by improving apoptosis of SNS fibers and marrow microenvironment cells.

We next imposed pharmacological Y1 receptor blockade in *Nes*-GFP mice (Méndez-Ferrer *et al*, 2010) by treating with the Y1 antagonist (BIBP3226) for 3 consecutive days, followed by treatment with 5FU (Supplementary Fig S2A). Y1 antagonist treatment caused reduction in mouse survival and hematopoietic function after 5FU injection (Supplementary Fig S2B and C and Supplementary Table S2). Moreover, we found reduced numbers of nestin-GFP$^+$ cells and CD31$^+$ ECs and increased apoptosis levels compared to control mice treated with 5FU (Supplementary Fig S2D and E). Of note, SNS fiber expression in BM was not changed by 5FU challenge in each group, similar to previous results (Supplementary Fig S2F and Lucas *et al*, 2013). Next, since ECs are known to express Y1 receptors (Kuo *et al*, 2007b), ECs cultured from BM were treated with the Y1 antagonist to confirm a direct role of the Y1 receptor in EC survival. The results showed increased apoptosis in ECs subjected to Y1 receptor blockade (Supplementary Fig S2G), indicating that the NPY/Y1 receptor system played a key role in the survival of HSCs and marrow microenvironment cells.

The NPY/Y1 receptor pathway in bone marrow injury

Chemotherapy causes acute bone marrow injury and impairs HSC function or bone marrow regeneration (Noach *et al*, 2000; Banfi *et al*, 2001; Lucas *et al*, 2013). In particular, chemotherapy drugs such as cisplatin and vincristine induce sympathetic neuropathy by reducing the expression of Th fibers (Lucas *et al*, 2013). Based on these concepts and our findings above, we predicted that NPY might protect against chemotherapy-induced neuropathy or bone marrow dysfunction through the Y1 receptor. To investigate this hypothesis, we first evaluated the expression of the Y1 receptor in major bone marrow microenvironment cells such as macrophages, osteoblasts, endothelial cells, and nestin$^+$ cells. Expression of Y1 receptor in macrophages and osteoblasts was increased compared to endothelial cells and nestin$^+$ cells (Fig 6A). Therefore, we focused on the Y1 receptor in these cells to study the mechanism of NPY-mediated BM recovery in chemotherapy-induced bone marrow injury. *Lyz2-cre* recombined in the myeloid cell lineage (Clausen *et al*, 1999), *Col1a1-cre* (Baek *et al*, 2009) and *Y1*$^{fl/fl}$ mice (Howell *et al*, 2003) was used to conditionally deplete Y1 receptor in macrophages and osteoblasts. Y1 receptor mRNA levels were markedly reduced in macrophages cultured from *Lyz2-cre*; *Y1*$^{fl/fl}$ mice and osteoblasts sorted from *Col1a1-cre*; *Y1*$^{fl/fl}$ mice, respectively (Fig 6B and C). Next, we treated control, *Lyz2-cre*; *Y1*$^{fl/fl}$, or *Col1a1-cre*; *Y1*$^{fl/fl}$ mice with seven cycles of cisplatin for the induction of neuropathy (Aloe *et al*, 2000). In some animals, NPY was administered during cisplatin chemotherapy (Fig 6D). No significant difference in the numbers of BMNCs was observed between the groups (Fig 6E). Of note, chemotherapy-induced HSC impairments were recovered in NPY-treated control or *Col1a1-cre*; *Y1*$^{fl/fl}$ mice (Fig 6F). In addition, NPY treatment improved the sensory neuropathy, reduced density of Th$^+$ fibers, and reduced the number of CD31$^+$ ECs in cisplatin-treated control or *Col1a1-cre*; *Y1*$^{fl/fl}$ mice after the bone marrow recovery period. Of interest, *Lyz2-cre*; *Y1*$^{fl/fl}$ mice did not show NPY-mediated protection of LT-HSCs, Th$^+$ fibers, and ECs due to the absence of Y1 receptor in macrophages (Fig 6G–I). Similar effects were observed in cisplatin-induced BM apoptosis (Fig 6J). These results suggested that NPY promoted neuroprotection and bone marrow dysfunction from cisplatin-induced injury through Y1 receptors in macrophages. Although previous reports have demonstrated that chemotherapy in patients with cancer affects the NPY levels (Cağlar *et al*, 2005), our data showed no significant difference in NPY expression of BM (Fig 6K).

To further confirm the possibility of neuroprotection by NPY through macrophage Y1 receptors, we cultured macrophages from the BM of control or *Lyz2-cre*; *Y1*$^{fl/fl}$ mice and treated these macrophages with NPY. Next, conditioned medium (CM) harvested from each group was applied to PC12 cells to determine the influence

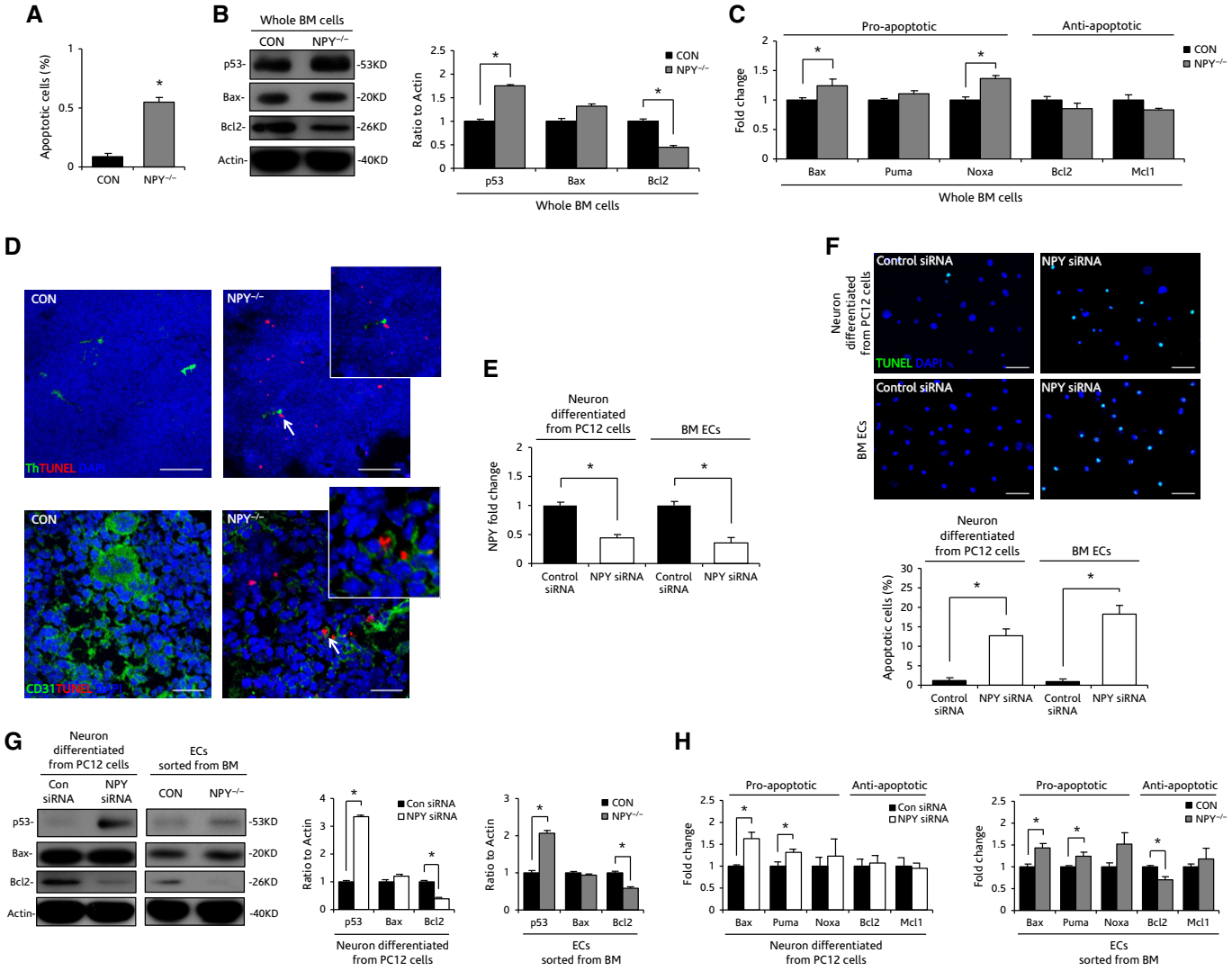

Figure 4. Reduction of SNS fibers and ECs is due to p53-dependent apoptosis.

A Quantification of apoptotic cells by TUNEL staining in BM of WT or NPY$^{-/-}$ mice (n = 6 mice per group).

B Western blot analysis and quantification for p53, Bax, and Bcl2 in whole BM cells of each group (n = 5).

C Quantitative real-time PCR analysis for pro- or anti-apoptotic genes expression in whole BM cells of each group (n = 5).

D Representative immunofluorescence images of BM showing apoptosis by TUNEL stain (red) in sympathetic neuron (Th, green, left; scale bar, 50 μm) or CD31^{+} ECs (green, right; scale bar, 20 μm).

E Expression levels of NPY in neurons and BM ECs exposed to control or NPY siRNA (n = 4).

F Left, representative immunofluorescence images of apoptotic neurons and BM ECs (green) exposed to control or NPY siRNA. Scale bar, 50 μm. Right, quantitation of the percentage of TUNEL-positive cells (n = 6 per group).

G Western blot analysis and quantification for p53, Bax, and Bcl2 expression in neuron differentiated from PC12 cells with control or NPY siRNA and BM ECs sorted from WT or NPY$^{-/-}$ (n = 4).

H Quantitative real-time PCR analysis for pro- or anti-apoptotic genes expression in BM of each group from (G) (n = 4).

Data information: *P < 0.05. All error bars indicate s.e.m. All expression levels are relative to Gapdh mRNA.
Source data are available online for this figure.

on neural differentiation. CM derived from NPY-treated control macrophages was able to induce differentiation of PC12 cells toward neurons, compared to CM derived from control macrophages. However, cells exposed to CM derived from NPY-treated Y1 receptor-deficient macrophages did not show neural differentiation capacity (Supplementary Fig S3A), indicating that neuroprotection by NPY is mediated by factors secreted from Y1 receptor-stimulated macrophages. Next, to reveal which factors released from macrophages induced neuroprotection, we screened and compared the CM of control or Y1 receptor-deficient macrophages with and without NPY for 50 different secreted cytokines using an antibody-based mouse cytokine array (Supplementary Fig S3B). The CM of control macrophages with NPY revealed stronger signals in 4 array spots in comparison with the CM of control macrophages alone (Supplementary

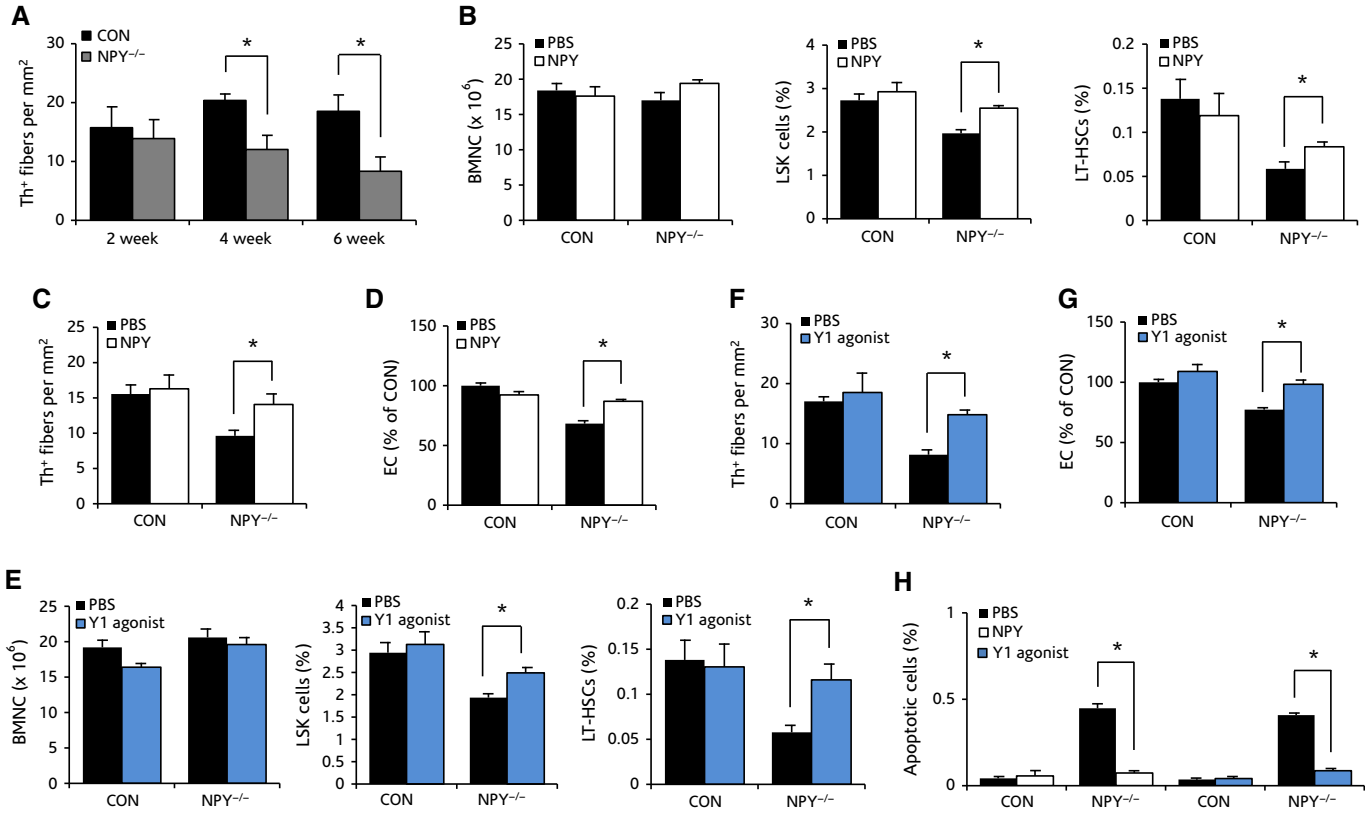

Figure 5. The NPY/Y1 receptor contributes to bone marrow microenvironment cell survival.

A Quantification of Th^+ fibers in the BM of 2-, 4-, and 6-week-old WT or $NPY^{-/-}$ mice (n = 6 mice per group).

B Number of BMNCs (left), percentage of LSK cells (middle), and LT-HSCs (right) in BM of WT or $NPY^{-/-}$ mice after NPY treatment for 4 weeks (n = 6 mice per group, i.p.).

C, D Quantification of (C) Th^+ fibers and number of (D) $CD31^+$ ECs in the BM of WT or $NPY^{-/-}$ mice after NPY treatment for 4 weeks (n = 6 mice per group).

E Number of BMNCs (left), percentage of LSK cells (middle), and LT-HSCs (right) in BM of WT or $NPY^{-/-}$ mice after Y1 agonist treatment for 4 weeks (n = 6 mice per group, i.p.).

F, G Quantification of (F) Th^+ fibers and number of (G) $CD31^+$ ECs in the BM of WT or $NPY^{-/-}$ mice after Y1 agonist treatment for 4 weeks (n = 6 mice per group).

H Percentage of apoptotic cells in BM of WT or $NPY^{-/-}$ mice after NPY or Y1 agonist treatment for 4 weeks (n = 6 mice per group).

Data information: $^*P < 0.05$. All error bars indicate s.e.m.

Fig S3C). We also confirmed the mRNA levels of these factors in control or Y1 receptor-deficient macrophages with or without NPY treatment and found up-regulated TGF-β expression in control macrophages with NPY. However, Y1 receptor-deficient macrophages with NPY did not evaluate TGF-β expression in both CM and cells (Supplementary Fig S3D and E).

TGF-β plays a key role in the regulation of neuronal survival, differentiation, and repair processes in the nervous system and is produced through the PI3K/Akt/mTOR/eIL4E signaling pathway (Krieglstein *et al*, 2002; Xiao *et al*, 2008; Knöferle *et al*, 2010; Vogel *et al*, 2010). Another study reported that NPY promoted TGF-β production by activating PI3K pathway via Y1 receptor (Zhou *et al*, 2008). Based on these previous studies and our data, we confirmed PI3K pathway in control or Y1 receptor-deficient macrophages treated with and without NPY. The results indicated that PI3K levels increased in control macrophages with NPY compared to control macrophages alone. Downstream signaling of PI3K pathway such as Akt/mTOR/eIL4E also was activated, but not in Y1 receptor-deficient macrophages treated with NPY (Supplementary Fig S3F). These findings suggested that TGF-β released by NPY-mediated Y1 receptor stimulation in macrophages promoted neuroprotection from cisplatin-induced injury. Recently, non-myelinated Schwann cells were shown to maintain stem cells in the bone marrow microenvironment by activating TGF-β. To confirm the possibility of neuroprotection by TGF-β secreted from Schwann cells, Schwann cells were cultured from the sciatic nerve of adult C57BL/6 mice (Supplementary Fig S4A). The cultured adult Schwann cells expressed Y1 receptors, although its expression was fewer than macrophages (Supplementary Fig S4B). TGF-β expression in NPY-treated Schwann cells showed no significant difference compare to control Schwann cells, as well as in CM (Supplementary Fig S4C and D). These results indicated that NPY/Y1 receptor in Schwann cells did not induce TGF-β secretion.

Next, to evaluate further whether NPY treatment prevents cisplatin-induced mouse death and impairment of BM, BMNCs were transplanted into cisplatin-treated mice with or without NPY treatment after lethal irradiation (Fig 7A). NPY treatment enhanced mouse survival, rescued bone marrow function after transplantation (Fig 7B and C and Supplementary Table S3), and also restored the

Published online: April 27, 2015
The EMBO Journal

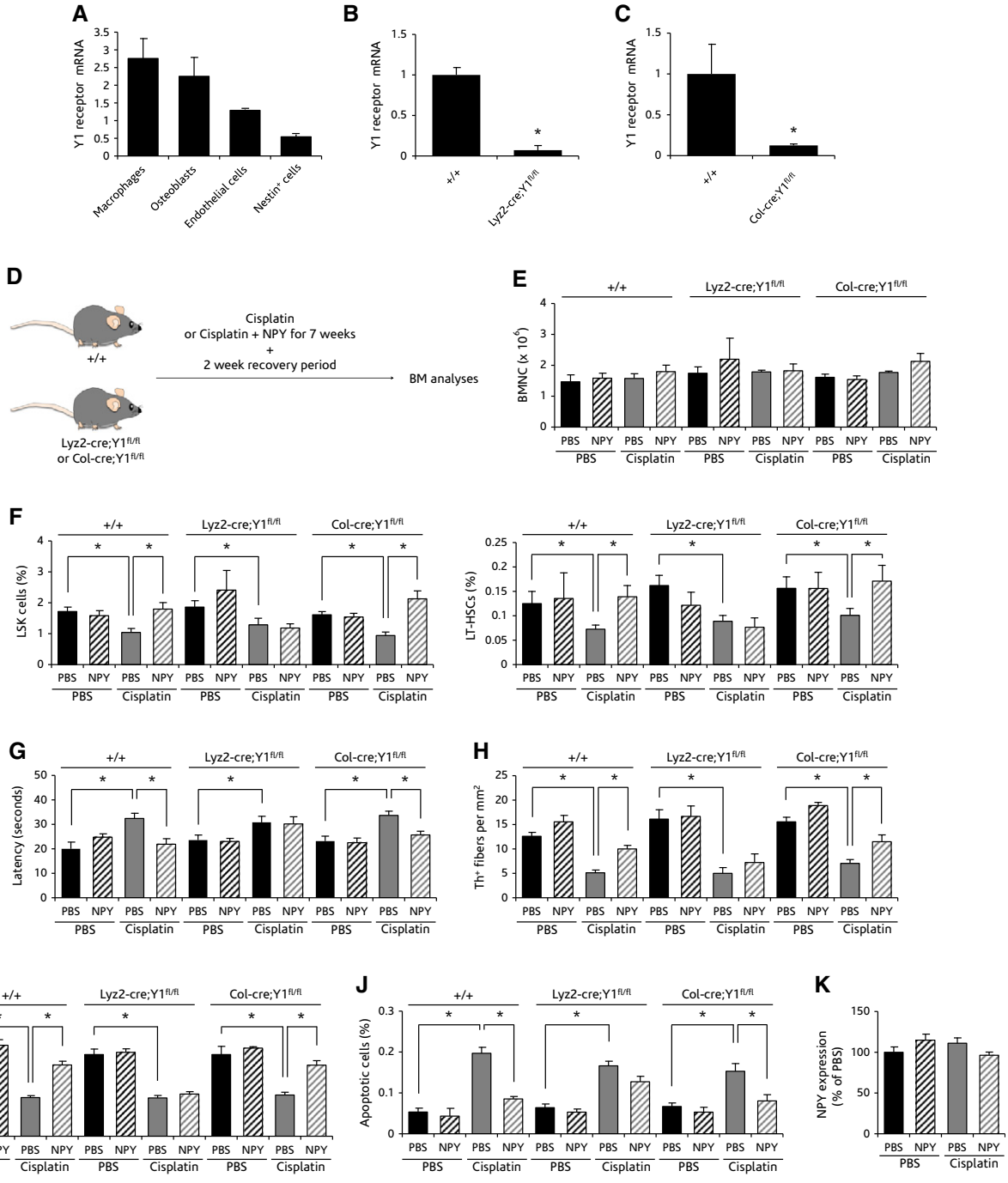

Figure 6. The NPY/Y1 receptor pathway in macrophages improves bone marrow injury.

A Expression of Y1 receptor in macrophages, osteoblasts, endothelial cells, and nestin$^+$ cells (n = 3 of cultured macrophage, and three experiments in which populations (osteoblasts, endothelial cells, and nestin$^+$ cells) were sorted from BM of ten mice each).

B, C Expression of Y1 receptor in (B) macrophages cultured from BM of WT or *Lyz2-cre*;*Y1*$^{fl/fl}$ mice (n = 5 mice per group) and (C) osteoblasts sorted from BM of WT or *Col1a1-cre*;*Y1*$^{fl/fl}$ mice (expression levels are the average of three experiments in which populations were sorted from ten mice each).

D Experimental design to determine the effect of Y1 receptor in macrophages or osteoblasts on cisplatin-induced neuropathy after NPY treatment.

E, F Number of (E) BMNCs, and (F) percentage of LSK cells and LT-HSCs of control, *Lyz2-cre*;*Y1*$^{fl/fl}$, or *Col1a1-cre*;*Y1*$^{fl/fl}$ mice treated as in (D) (n = 4–6 mice per group).

G Quantification of sensory neuropathy (2 weeks after the last cisplatin injection) in control, *Lyz2-cre*;*Y1*$^{fl/fl}$, or *Col1a1-cre*;*Y1*$^{fl/fl}$ mice treated with cisplatin alone or with cisplatin and NPY (n = 6–10 mice per group, i.p.). Latency indicates the amount of time until the mouse showed signs of nociception (jumping or paw licking).

H, I Quantification of (H) Th$^+$ fibers and number of (I) CD31$^+$ ECs in the BM of control, *Lyz2-cre*;*Y1*$^{fl/fl}$, or *Col1a1-cre*;*Y1*$^{fl/fl}$ mice treated as in (D) (n = 5–6 mice per group).

J Percentage of apoptotic cells in BM of control, *Lyz2-cre*;*Y1*$^{fl/fl}$, or *Col1a1-cre*;*Y1*$^{fl/fl}$ mice treated as in (D) (n = 5–6 mice per group).

K Quantification of NPY expression in the BM of control, *Lyz2-cre*;*Y1*$^{fl/fl}$, or *Col1a1-cre*;*Y1*$^{fl/fl}$ mice treated as in (D) (n = 5–6 mice per group).

Data information: *P < 0.05. All error bars indicate s.e.m. All expression levels are relative to Gapdh mRNA. +/+, wild-type.

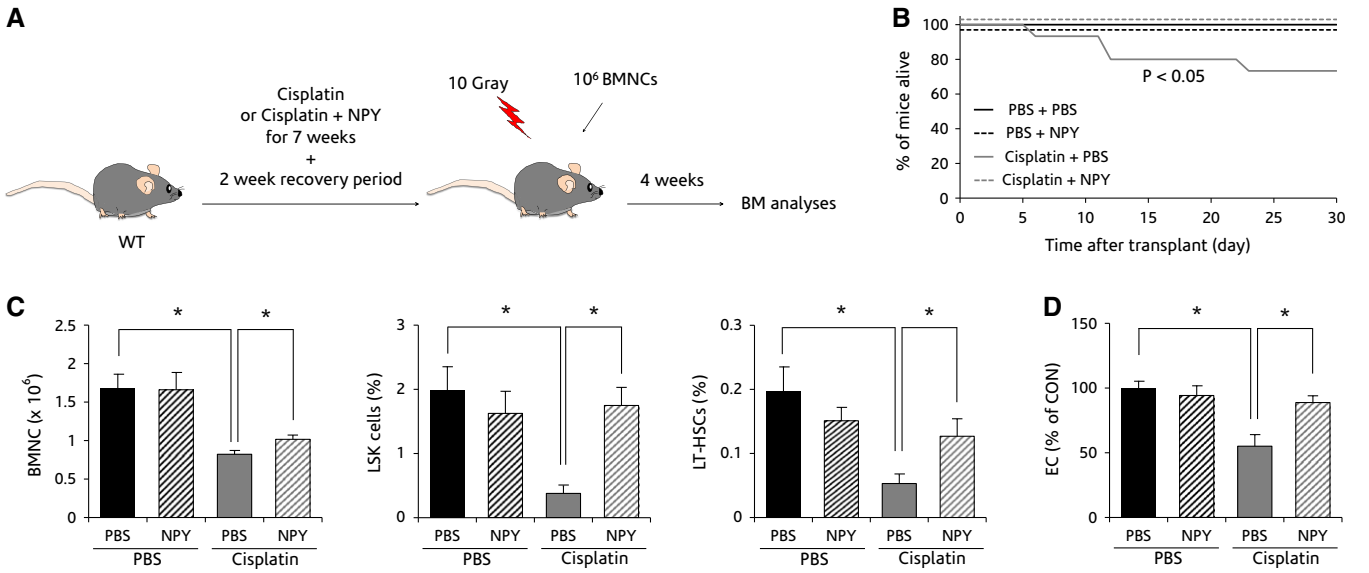

Figure 7. NPY prevents cisplatin-induced mouse death by restoring bone marrow dysfunction.

A Experimental design to determine whether NPY protects bone marrow microenvironment cells from cisplatin-induced bone marrow impairment and accelerates bone marrow recovery after transplantation.

B Survival of WT mice treated with PBS, cisplatin, NPY, or cisplatin + NPY (n = 10–15 mice per group).

C Number of BMNCs (left), and percentage of LSK cells (middle) and LT-HSCs (right) in BM of WT mice treated with PBS, cisplatin, NPY, or cisplatin + NPY as assessed 4 weeks after bone marrow transplantation (n = 5–6 mice per group).

D Percentage of $CD31^+$ ECs per femur in WT mice treated with PBS, cisplatin, NPY, or cisplatin + NPY (n = 5–6 mice per group).

Data information: $^*P < 0.05$. All error bars indicate s.e.m.

numbers of $CD31^+$ ECs in cisplatin-treated mice (Fig 7D). Collectively, our findings strongly support the concept that NPY is required for the maintenance of bone marrow function by protecting SNS fibers and bone marrow microenvironment cell survival through TGF-β secreted from Y1 receptors in macrophages, offering a new potential therapeutic intervention for chemotherapy-induced bone marrow impairment.

Discussion

Recent studies have suggested that the bone marrow environment is regulated by the SNS (Katayama *et al*, 2006; Méndez-Ferrer *et al*, 2008; Yamazaki *et al*, 2011; Lucas *et al*, 2013). However, the mechanisms through which neurotransmitters released from the SNS regulate bone marrow microenvironments remain largely unknown. Here we elucidate an unknown function of NPY in regulating the bone marrow HSC microenvironment. NPY-deficient mice displayed significantly decreased numbers of HSCs and impaired regeneration in BM after bone marrow transplantation, although these same mice exhibited no effect on homing of hematopoietic progenitors to the BM. Previous studies reported that NPY is produced by sympathetic nerve fibers, as well as bone marrow hematopoietic cells (Wheway *et al*, 2005; Kuo *et al*, 2007b). To determine the possibility of bone marrow dysfunction by NPY-deficient marrow cells, we transplanted WT or $NPY^{-/-}$ BMNCs into lethally irradiated WT mice. The results showed no significant difference in mouse survival, repopulating efficiency in BM of WT or $NPY^{-/-}$ BMNCs transplanted recipient mice, as well as homing to BM. Moreover, reduced HSC numbers in BM of NPY-deficient mice were not due to a cell-autonomous effect by NPY absence. These results demonstrated that bone marrow microenvironment cells were related to HSC survival in NPY-deficient mice, further supporting the concept that NPY produced by sympathetic nerve fibers was required for HSC survival and bone marrow regeneration. NPY-deficient mice also exhibited reduced MSCs, suggesting that NPY may mediate MSC survival. More studies are required to determine the specific role of NPY in the regulation of BM MSCs.

In this study, NPY deficiency caused destruction of SNS fibers and/or a decrease in the number of ECs preserving HSC survival. Moreover, NPY absence or Y1 receptor inhibition in ECs directly affected ECs apoptosis. Notably, the reductions of SNS fibers and bone marrow microenvironment cells were protected by NPY or Y1 agonist injection in $NPY^{-/-}$ mice. In contrast, inhibition of the Y1 receptor showed impairment of HSCs and bone marrow microenvironment cell survival after 5FU treatment. Overall, these results support the notion that an interaction of NPY and the Y1 receptor could mediate the survival of HSCs residing in the perivascular microenvironment by the regulation of bone marrow SNS fibers, and that this interaction maintains survival of ECs and $nestin^+$ cells, which in turn is associated with HSC retention (Supplementary Fig S5). Recently, a very small population of αSMA-expressing macrophages also was shown to maintain BM HSPCs under steady state (Ludin *et al*, 2012). However, this cell population was not changed in BM of $NPY^{-/-}$ mice, as well as other macrophage subsets such as $CD11b^+F4/80^+Ly6G^+$ and $F4/80^+CD115^+Gr1^-CD169^+$. These results indicated that NPY deficiency did not affect HSCs maintained by macrophages.

Chemotherapy-induced neuropathy is an important side effect of cancer therapy. In addition, chronic bone marrow damage by chemotherapy accompanies impaired hematopoietic regeneration, and this leads to reduced recovery of bone marrow microenvironment cells (Cavaletti & Marmiroli, 2010; Lucas *et al*, 2013). In this study, we found that NPY treatment restored bone marrow function in cisplatin-treated mice. These results suggest that NPY warrants further study regarding its potential therapeutic use for chemotherapy-induced bone marrow abnormalities. Additionally, our findings indicated that cisplatin-induced SNS injury was prevented by NPY treatment, which reduced cisplatin-induced apoptosis of BM cells. This protective effect was not found in macrophages of Y1 receptor-deficient mice. *In vitro* experiment evaluating PC12 cell differentiation toward neurons found that PC12 cells exposed to CM derived from NPY-treated control macrophages showed neural differentiation capacity, but not in Y1 receptor-deficient macrophages treated with NPY. These results highlight the fact that trophic or bioactive factors secreted from macrophages through NPY/Y1 regulation mediates neural protection. We also found up-regulated TGF-β secretion or expression by NPY treatment. The PI3K/Akt/mTOR/eIL4E signaling pathway that produces TGF-β also was activated in macrophages with NPY. These findings are supported by previous studies showing that TGF-β promotes neuronal survival and differentiation (Krieglstein *et al*, 2002; Xiao *et al*, 2008; Knöferle *et al*, 2010; Vogel *et al*, 2010). TGF-β released from non-myelinated Schwann cells or extracellular matrix in bone marrow has been shown to maintain stem cell survival or recruiting (Wu *et al*, 2010; Yamazaki *et al*, 2011). We confirmed expression of Y1 receptor in Schwann cells; however, NPY treatment did not increase TGF-β secretion from Schwann cells. Taken together, these results indicated that NPY-induced TGF-β activation in macrophages is responsible for neuroprotection and HSC survival in bone marrow. Further studies are needed to reveal additional roles of NPY/Y1 receptor regulated TGF-β secretion from macrophages on neural protection in the bone marrow microenvironment.

Our findings provide proof-of-concept that NPY or Y1 agonists have a potential clinical utility as neuroprotective agents for patients treated with chemotherapy. Importantly, NPY has the clinical benefit of being a stable peptide that is synthesized naturally or can be made easily through recombinant technology. Therefore, the modulation of the endogenous bone marrow HSC microenvironment by NPY could be a more effective therapeutic approach than direct transplantation using BM or stem cells. These findings also suggest that future studies investigating the role of NPY in other tissue stem cell microenvironments (for example, the adult neural stem cell microenvironment) may provide new insights in the fields of stem cell research and cell therapy.

Materials and Methods

Mice

Six- to eight-week-old male or female $NPY^{-/-}$ mice, C57BL/6 mice, or C57BL/6.SJL (BoyJ) mice were purchased from the Jackson Laboratory. *Nes*-GFP mice (Méndez-Ferrer *et al*, 2010) have been described previously. $NPY^{-/-}$ mice and control mice were maintained on a 129S1/SvImJ background. *Lyz2-cre* (Clausen *et al*, 1999) (The Jackson Laboratory), 2.3-kB *Col1a1-cre* (Baek *et al*, 2009), and $Y1^{fl/fl}$ mice (Howell *et al*, 2003) were used to delete the Y1 receptor in osteoblasts and macrophages specifically. The block randomization method was used to allocate the animals to experimental group. To eliminate bias, investigators were blinded during data collection and data analysis. Mice were housed at a 12-h day–night cycle with free access to tap water and food pellets. All mouse studies were approved by the Kyungpook National University Institutional Animal Care and Use Committee.

Reagent treatments

To induce bone marrow ablation and force proliferation of quiescent HSCs, we injected 5FU (Sigma; 250 mg per kg body weight, i.v.) under isoflurane anesthesia. Cisplatin (Enzo; 10 mg per kg body weight once per week) was used to induce SNS injury, and mice received i.p. injections of cisplatin for 7 weeks as described (Aloe *et al*, 2000). One hour after the 7^{th} injection, some mice were euthanized for analysis; 2 weeks after the last injection of cisplatin (to allow for full bone marrow recovery), mice were tested for sensory neuropathy. To assess neuroprotection from cisplatin, mice were injected i.p. with NPY (Bachem, H-6375) daily during the 7-week cisplatin treatment period. To investigate the role of the Y1 receptor in the bone marrow microenvironment, 10 nM NPY (Bachem, H-6375) was applied to macrophages *in vitro*. Three days later, CM from macrophages was collected. Cultured PC12 cells (Korean cell line bank, 21721) in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin (all from Gibco) were exposed to CM derived from NPY (10 nM)-treated macrophages to determine its influence on differentiation. Seven days later, the percentage of PC12 cells with two or more dendrites was scored under an Olympus IX71 microscope. NPY siRNA (GE Dharmacon) was treated in LSK cells, neurons differentiated from PC12 cells, and BM ECs and osteoblasts differentiated from MC3T3-E1 cells (ATCC, CRL-2594) for 48 h. For *in vivo* experiments, $NPY^{-/-}$ or *Nes*-GFP mice were injected with NPY, Y1 agonist, or Y1 antagonist (50 μg per kg body weight, i.v.). After 1 h, the blood and BM were harvested and analyzed.

Bone marrow transplantation

For assessment of bone marrow microenvironment function of $NPY^{-/-}$ mice, mice were lethally irradiated (10 Gy, two split doses) and injected with 1×10^6 BM from CD45.1 (BoyJ) mice. In some experiments, lethally irradiated CD45.1 mice were transplanted by 1×10^6 BM cells from $NPY^{-/-}$ mice and control mice intravenously. Eight weeks later, the percentage of donor-derived cells present in the blood was measured by flow cytometry. For homing assays, lethally irradiated WT and $NPY^{-/-}$ mice were injected with 5×10^6 BM from $CD45.1^+$ (BoyJ) mice. After 24 h, the mice were sacrificed, and bone marrow cells were harvested and assayed for competitive transplantation or the presence of CFU-C as described (Miller *et al*, 2008). For competitive transplantation, BM collected from the tibiae and femurs of recipient mice and 10^6 $CD45.2^+$ BM of C57BL/6 were transplanted into lethally irradiated second recipient mice; sixteen weeks after transplantation, the percentage of $CD45.1^+$ cells in the blood of the recipient mice was quantified (Yuan *et al*, 2005).

Flow cytometry

BM was flushed from the tibiae and femurs of each mouse. Red blood cells (RBCs) were lysed once for 5 min at 4°C in 0.15 M NH_4Cl (StemCell Technologies), washed once with PBS (Gibco), and counted using a hemocytometer. For HSC, MSC, or osteoblast detection, Lin^+ cells were removed by magnetic depletion using biotinylated lineage-specific antibodies (CD5, CD45R, CD11b, Gr-1, and Ter-119), followed by depletion with MACs beads conjugated to a monoclonal anti-biotin (Miltenyi Biotec). For staining of HSCs, Lin^- cells were stained with phycoerythrin PE-Cy7-conjugated antibodies to Sca1 (558162), APC-conjugated antibodies to c-Kit (553356), FITC-conjugated antibodies to CD48 (557484), and PE-conjugated antibodies to CD150 (561540), all from BD Biosciences. For MSCs and osteoblasts staining, the chopped bone fragments and BM were digested with collagenase type I (3 mg/ml; Sigma), and Lin^- cells were stained with APC-Cy7-CD45 (557659), APC-CD31 (551262), PE-Cy7-Sca1 (558162), or PE-CD51 (551187), all from BD Biosciences. Cells were further stained with streptavidin-pacific blue (PB) (Invitrogen, S11222). Osteoblasts were sorted. Macrophages subsets, T cells, and B cells were detected by staining with antibodies to PE-CD11b (BD Biosciences, 557397), FITC-Ly6G (BD Biosciences, 551160), APC-F4/80 (AbD Serotec, MCA497), APC cy7-Gr1 (BD Biosciences, 557661), FITC-CD169 (AbD Serotec, MCA884F), PE-CD115 (eBioscience, 12-1152), αSMA (Abcam, ab66133), PE-CD3 (BD Biosciences, 555275), and PB-B220 (BD Biosciences, 558108). Data were collected on a BD LSRII system and AriaIII (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Immunofluorescence staining of bone marrow sections

Frozen bone marrow sections were prepared and immunostained according to a previously published method (Kawamoto, 2003). Bone marrow sections were fixed using dry ice/hexane. Sections were incubated with one primary antibody followed by incubation with secondary antibody conjugated with either Alexa488 or Alexa594 (Life Technologies). Immunofluorescence data were obtained and analyzed using a laser scanning confocal microscope equipped with Fluoview SV1000 imaging software (Olympus FV1000; Japan). Antibodies used were as follows: NPY (Abcam, ab30914, 1:250 dilution), Th (Millipore, AB152 or AB318, 1:250 dilution), and CD31 (BD Biosciences, 550300, 1:50 dilution). TUNEL assay was performed using the *In Situ* Cell Detection Kit, TMR red (Roche) following the manufacturer's instructions.

Statistical analysis

Comparisons between two groups were performed with Student's *t*-test. In cases where more than two groups were compared to each other, a one-way analysis of variance (ANOVA) was used, followed by Tukey's HSD test. Comparisons of overall survival were performed using a log-rank test. All statistical analyses were performed using SPSS statistical software. $P < 0.05$ was considered to be significant.

Supplementary information for this article is available online: http://emboj.embopress.org

Acknowledgements
This work was supported by the Bio & Medical Technology Development Program (2012M3A9C6049913) and Basic Science Research Program (2014R1A2A1A10051107) of the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning, Republic of Korea. This research was also supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (HI14C1636).

Author contributions
MHP and HKJ designed and performed the experiments and wrote the paper. WKM, WWL, and JEL performed the experiments and analyzed the data. EHS and JSB interpreted the data and reviewed the paper. JSB designed the study and wrote the paper. HA, HH, and GNE generated and provided *Col1a1-cre*, $Y1^{fl/fl}$, and *Nes*-GFP mice.

Conflict of interest
The authors declare that they have no conflict of interest.

References

Aloe L, Manni L, Properzi F, De Santis S, Fiore M (2000) Evidence that nerve growth factor promotes the recovery of peripheral neuropathy induced in mice by cisplatin: behavioral, structural and biochemical analysis. *Auton Neurosci* 86: 84 – 93

Amiya E, Watanabe M, Komuro I (2014) The relationship between vascular function and the autonomic nervous system. *Ann Vasc Dis* 7: 109 – 119

Arai F, Hirao A, Ohmura M, Sato H, Matsuoka S, Takubo K, Ito K, Koh GY, Suda T (2004) Tie2/angiopoietin-1 signaling regulates hematopoietic stem cells quiescence in the bone marrow niche. *Cell* 118: 149 – 161

Baek WY, Lee MA, Jung JW, Kim SY, Akiyama H, de Crombrugghe B, Kim JE (2009) Positive regulation of adult bone formation by osteoblast-specific transcription factor osterix. *J Bone Miner Res* 24: 1055 – 1065

Banfi A, Podestà M, Fazzuoli L, Sertoli MR, Venturini M, Santini G, Cancedda R, Quarto R (2001) High-dose chemotherapy shows a dose-dependent toxicity to bone marrow osteoprogenitors: a mechanism for post-bone marrow transplantation osteopenia. *Cancer* 92: 2419 – 2428

Butler JM, Nolan DJ, Vertes EL, Varnum-Finney B, Kobayashi H, Hooper AT, Seandel M, Shido K, White IA, Kobayashi M, Witte L, May C, Shawber C, Kimura Y, Kitajewski J, Rosenwaks Z, Bernstein ID, Rafii S (2010) Endothelial cells are essential for the self-renewal and repopulation of Notch-dependent hematopoietic stem cells. *Cell Stem Cell* 6: 251 – 264

Cağlar K, Kutluk T, Varan A, Koray Z, Akyüz C, Yalçin B, Erbaş B, Büyükpamukçu M (2005) Leptin and neuropeptide Y plasma levels in children with cancer. *J Pediatr Endocrinol Metab* 18: 485 – 489

Cavaletti G, Marmiroli P (2010) Chemotherapy-induced peripheral neurotoxicity. *Nat Rev Neurol* 6: 657 – 666

Chow A, Lucas D, Hidalgo A, Méndez-Ferrer S, Hashimoto D, Scheiermann C, Battista M, Leboeuf M, Prophete C, van Rooijen N, Tanaka M, Merad M, Frenette PS (2011) Bone marrow CD169+ macrophages promote the retention of hematopoietic stem and progenitor cells in the mesenchymal stem cell niche. *J Exp Med* 208: 261 – 271

Clausen BE, Burkhardt C, Reith W, Renkawitz R, Förster I (1999) Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic Res* 8: 265 – 277

Corvino V, Marchese E, Giannetti S, Lattanzi W, Bonvissuto D, Biamonte F, Mongiovì AM, Michetti F, Geloso MC (2012) The neuroprotective and

neurogenic effects of neuropeptide Y administration in an animal model of hippocampal neurodegeneration and temporal lobe epilepsy induced by trimethyltin. *J Neurochem* 122: 415 – 426

Ding L, Saunders TL, Enikolopov G, Morrison SJ (2012) Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature* 481: 457 – 462

Doan PL, Himburg HA, Helms K, Russell JL, Fixsen E, Quarmyne M, Harris JR, Deoliviera D, Sullivan JM, Chao NJ, Kirsch DG, Chute JP (2013) Epidermal growth factor regulates hematopoietic regeneration after radiation injury. *Nat Med* 19: 295 – 304

Ehninger A, Trumpp A (2011) The bone marrow stem cell niche grows up mesenchymal stem cells and macrophages move in. *J Exp Med* 208: 421 – 428

Gonçalves J, Ribeiro CF, Malva JO, Silva AP (2012) Protective role of neuropeptide Y Y_2 receptors in cell death and microglial response following methamphetamine injury. *Eur J Neurosci* 36: 3173 – 3183

Hong HS, Lee J, Lee E, Kwon YS, Lee E, Ahn W, Jiang MH, Kim JC, Son Y (2009) A new role of substance P as an injury-inducible messenger for mobilization of CD29+ stromal-like cells. *Nat Med* 15: 425 – 435

Howell OW, Scharfman HE, Herzog H, Sundstrom LE, Beck-Sickinger A, Gray WP (2003) Neuropeptide Y is neuroproliferative for post-natal hippocampal precursor cells. *J Neurochem* 86: 646 – 659

Kalra SP, Kalra PS (2004) NPY and cohorts in regulation appetite, obesity and metabolic syndrome: beneficial effects of gene therapy. *Neuropeptides* 38: 201 – 211

Katayama Y, Battista M, Kao WM, Hidalgo A, Peired AJ, Thomas SA, Frenette PS (2006) Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. *Cell* 124: 407 – 421

Kawamoto T (2003) Use of a new adhesive film for the preparation of multi-purpose fresh-frozen sections from hard tissues, whole-animals, insects and plants. *Arch Histol Cytol* 66: 123 – 143

Knöferle J, Ramljak S, Koch JC, Tönges L, Asif AR, Michel U, Wouters FS, Heermann S, Krieglstein K, Zerr I, Bähr M, Lingor P (2010) TGF-beta 1 enhances neurite outgrowth via regulation of proteasome function and EFABP. *Neurobiol Dis* 38: 395 – 404

Krieglstein K, Strelau J, Schober A, Sullivan A, Unsicker K (2002) TGF-beta and the regulation of neuron survival and death. *J Physiol Paris* 96: 25 – 30

Kuo LE, Abe K, Zukowska Z (2007b) Stress, NPY and vascular remodeling: implications for stress-related diseases. *Peptides* 28: 435 – 440

Kuo LE, Kitlinska JB, Tilan JU, Li L, Baker SB, Johnson MD, Lee EW, Burnett MS, Fricke ST, Kvetnansky R, Herzog H, Zukowska Z (2007a) Neuropeptide Y acts directly in the periphery on fat tissue and mediates stress-induced obesity and metabolic syndrome. *Nat Med* 13: 803 – 811

Lapidot T, Dar A, Kollet O (2005) How do stem cells find their way home? *Blood* 106: 1901 – 1910

Lee NJ, Herzog H (2009) NPY regulation of bone remodeling *Neuropeptides*43: 457 – 463

Lévesque JP, Helwani FM, Winkler IG (2010) The endosteal 'osteoblastic' niche and its role in hematopoietic stem cell homing and mobilization. *Leukemia* 24: 1979 – 1992

Lucas D, Scheiermann C, Chow A, Kunisaki Y, Bruns I, Barrick C, Tessarollo L, Frenette PS (2013) Chemotherapy-induced bone marrow nerve injury impairs hematopoietic regeneration. *Nat Med* 19: 695 – 703

Ludin A, Itkin T, Gur-Cohen S, Mildner A, Shezen E, Golan K, Kollet O, Kalinkovich A, Porat Z, D'Uva G, Schajnovitz A, Voronov E, Brenner DA, Apte RN, Jung S, Lapidot T (2012) Monocytes-macrophages that express α-smooth muscle actin preserve primitive hematopoietic cells in the bone marrow. *Nat Immunol* 13: 1072 – 1082

Méndez-Ferrer S, Lucas D, Battista M, Frenette PS (2008) Haematopoietic stem cell release is regulated by circadian oscillations. *Nature* 452: 442 – 447

Méndez-Ferrer S, Michurina TV, Ferraro F, Mazloom AR, Macarthur BD, Lira SA, Scadden DT, Ma'ayan A, Enikolopov GN, Frenette PS (2010) Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* 466: 829 – 834

Miller CL, Dykstra B, Eaves CJ (2008) Characterization of mouse hematopoietic stem and progenitor cells. *Curr Protoc Immunol* 80: 22B.2

Noach EJ, Ausema A, van Os R, Akkerman I, Koopal S, Weersing E, Dontje B, Vellenga E, de Haan G (2000) Chemotherapy prior to autologous bone marrow transplantation impairs long-term engraftment in mice. *Exp Hematol* 28: 1325 – 1333

Scadden DT (2006) The stem-cell niche as an entity of action. *Nature* 441: 1075 – 1079

Spiegel A, Shivtiel S, Kalinkovich A, Ludin A, Netzer N, Goichberg P, Azaria Y, Resnick I, Hardan I, Ben-Hur H, Nagler A, Rubinstein M, Lapidot T (2007) Catecholaminergic neurotransmitters regulate migration and repopulation of immature human CD34+ cells through Wnt signaling. *Nat Immunol* 8: 1123 – 1131

Sugiyama T, Kohara H, Noda M, Nagasawa T (2006) Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity* 25: 977 – 988

Trimmer PA, Smith TS, Jung AB, Bennett JP Jr (1996) Dopamine neurons from transgenic mice with a knockout of the p53 gene resist MPTP neurotoxicity. *Neurodegeneration* 5: 233 – 239

Vogel T, Ahrens S, Büttner N, Krieglstein K (2010) Transforming growth factor beta promotes neuronal cell fate of mouse cortical and hippocampal progenitors in vitro and in vivo: identification of Nedd9 as an essential signaling component. *Cereb Cortex* 20: 661 – 671

Wheway J, Mackay CR, Newton RA, Sainsbury A, Boey D, Herzog H, Mackay F (2005) A fundamental bimodal role for neuropeptide Y1 receptor in the immune system. *J Exp Med* 202: 1527 – 1538

Winkler IG, Sims NA, Pettit AR, Barbier V, Nowlan B, Helwani F, Poulton IJ, van Rooijen N, Alexander KA, Raggatt LJ, Lévesque JP (2010) Bone marrow macrophages maintain hematopoietic stem cell (HSC) niches and their depletion mobilizes HSCs. *Blood* 116: 4815 – 4828

Wu X, Pang L, Lei W, Lu W, Li J, Li Z, Frassica FJ, Chen X, Wan M, Cao X (2010) Inhibition of Sca-1-positive skeletal stem cell recruitment by alendronate blunts the anabolic effects of parathyroid hormone on bone remodeling. *Cell Stem Cell* 7: 571 – 580

Xiao YQ, Freire-de-Lima CG, Schiemann WP, Bratton DL, Vandivier RW, Henson PM (2008) Transcriptional and translational regulation of TGF-beta production in response to apoptotic cells. *J Immunol* 181: 3575 – 3585

Yamazaki S, Ema H, Karlsson G, Yamaguchi T, Miyoshi H, Shioda S, Taketo MM, Karlsson S, Iwama A, Nakauchi H (2011) Nonmyelinating schwann cells maintain hematopoietic stem cell hibernation in the bone marrow niche. *Cell* 147: 1146 – 1158

Yuan R, Astle CM, Chen J, Harrison DE (2005) Genetic regulation of hematopoietic stem cell exhaustion during development and growth. *Exp Hematol* 33: 243 – 250

Zhou JR, Xu Z, Jiang CL (2008) Neuropeptide Y promotes TGF-beta1 production in RAW264.7 cells by activating PI3K pathway via Y1 receptor. *Neurosci Bull* 24: 155 – 159

Zukowska-Grojec Z (1995) Neuropeptide Y. A novel sympathetic stress hormone and more. *Ann NY Acad Sci* 771: 219 – 233

