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Neuropeptide Y Induces Hematopoietic Stem/Progenitor Cell Mobilization by Regulating Matrix Metalloproteinase-9 Activity Through Y1 Receptor in Osteoblasts

MIN HEE PARK,[a,b,c] JONG KIL LEE,[a,b,c] NAMOH KIM,[a,b,c] WOO-KIE MIN,[d] JEONG EUN LEE,[e] KYOUNG-TAE KIM,[f] HARUHIKO AKIYAMA,[g] HERBERT HERZOG,[h] EDWARD H. SCHUCHMAN,[i] HEE KYUNG JIN,[a,j] JAE-SUNG BAE[a,b,c]

[a]Stem Cell Neuroplasticity Research Group, Kyungpook National University; [b]Department of Physiology, Cell and Matrix Research Institute, School of Medicine; [c]Department of Biomedical Science, BK21 Plus KNU Biomedical Convergence Program; [d]Department of Orthopaedic Surgery; [e]Department of Radiation Oncology; [f]Department of Neurosurgery School of Medicine; [j]Department of Laboratory Animal Medicine, College of Veterinary Medicine, Kyungpook National University, Daegu, Korea; [g]Department of Orthopaedics, Kyoto University, Kyoto, Japan; [h]Neuroscience Research Program, Neuroscience Division, Garvan Institute of Medical Research, Sydney, Australia; [i]Department of Genetics and Genomic Sciences, Icahn School of Medicine at Mount Sinai, New York, New York, USA

Correspondence: Jae-sung Bae, D.V.M., Ph.D., School of Medicine, Kyungpook National University, 680 Gukchaebosang-ro, Jung-gu, Daegu 700-842, South Korea. Telephone: + 82 53 420 4815; Fax: + 82 53 424 3349; e-mail: jsbae@knu.ac.kr; or Hee Kyung Jin, D.V.M., Ph.D., College of Veterinary Medicine, Kyungpook National University, 80 Daehakro, Buk-gu, Daegu, 702-701, South Korea. Telephone: + 82 53 950 5966; Fax: + 82 53 950 5955; e-mail: hkjin@knu.ac.kr

M.H.P.: and J.K.L. contributed equally to this article.

Received October 2, 2015; accepted for publication March 26, 2016; first published online in STEM CELLS *EXPRESS* April 19, 2016.

1066-5099/2016/$30.00/0

http://dx.doi.org/10.1002/stem.2383

Key Words. Neuropeptide Y • Bone marrow environment • Hematopoietic stem/progenitor cell • Mobilization • Bone loss

ABSTRACT

Hematopoietic stem/progenitor cell (HSPC) mobilization is an essential homeostatic process regulated by the interaction of cellular and molecular components in bone marrow niches. It has been shown by others that neurotransmitters released from the sympathetic nervous system regulate HSPC egress from bone marrow to peripheral blood. In this study, we investigate the functional role of neuropeptide Y (NPY) on this process. NPY deficient mice had significantly impaired HSPC mobilization due to increased expression of HSPC maintenance factors by reduction of matrix metalloproteinase-9 (MMP-9) activity in bone marrow. Pharmacological or endogenous elevation of NPY led to decrease of HSPC maintenance factors expression by activating MMP-9 in osteoblasts, resulting in HSPC mobilization. Mice in which the Y1 receptor was deleted in osteoblasts did not exhibit HSPC mobilization by NPY. Furthermore, NPY treatment in ovariectomized mice caused reduction of bone loss due to HSPC mobilization. These results suggest a new role of NPY on HSPC mobilization, as well as the potential therapeutic application of this neuropeptide for stem cell-based therapy. STEM CELLS *2016;34:2145–2156*

SIGNIFICANCE STATEMENT

Neurotransmitters released from the SNS regulate HSPC mobilization. NPY is one of the most abundant and widely secreted peptides from the brain or sympathetic nerves, and it has been implicated in a variety of physiological actions. However, the specific function of NPY in the HSPC mobilization has not been fully characterized. Our results suggest that NPY induces HSPC mobilization through Y1 receptor in osteoblasts by activating MMP-9. Moreover, NPY induced HSPC mobilization improves bone loss of ovariectomized mice, suggesting new role of NPY as a mediator of HSPC mobilization and potential therapeutic agent for bone loss.

INTRODUCTION

Hematopoietic stem/progenitor cells (HSPCs) residing in bone marrow niches are responsible for the regeneration and repopulation of all blood cell lineages, and lifelong hematopoiesis [1]. The bone marrow niches that anchor HSPCs are a highly organized microenvironment, and consist of supporting cells that regulate HSPCs survival, self-renewal, proliferation, and mobilization [2–5]. Osteoblasts in the endosteal niche express various hematopoietic cytokines and adhesion molecules, such as SDF-1α, Kitl, Angpt1, and Vcam1 [6–9], which in turn regulate HSPC homeostasis and egress from bone marrow (BM) [10–12]. Nestin$^+$ mesenchymal stem cells (MSCs) within the perivascular niche also express adhesion molecules mediating the retention and mobilization of HSPCs [13, 14].

Mobilization is one of the most fundamental properties of HSPCs, and occurs through interaction between HSPCs and cellular/molecular components in BM [15, 16]. Recently, the nervous system has been shown to affect the mobilization network in BM. For example, acute variation of the sympathetic nervous system (SNS) leads to HSPC egress by reducing SDF-1α expression in MSCs. Moreover, sympathetic nerve fibers storing large amount of neurotransmitters also are important regulators of HSPC mobilization [17–21]. In addition,

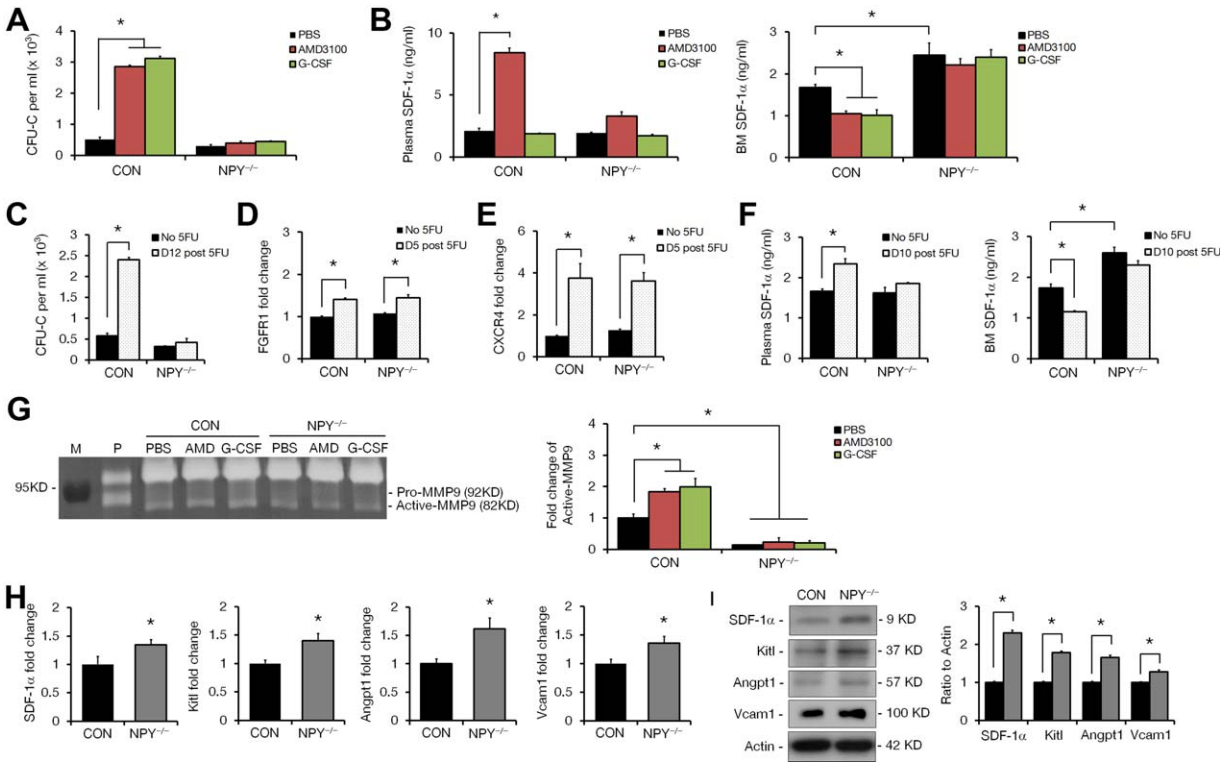

Figure 1. NPY deficiency impairs HSPC mobilization. **(A)**: Number of CFU-C in PB of WT or NPY$^{-/-}$ mice mobilized with AMD3100 or G-CSF (n = 4–6 mice per group). **(B)**: SDF-1α levels in plasma and BM supernatants after treatment of AMD3100 or G-CSF (n = 3–4 mice per group). **(C)**: Number of CFU-C in PB of WT or NPY$^{-/-}$ mice mobilized with 5FU (n = 5 mice per group). **(D, E)**: Expression of (D) FGFR1 and (E) CXCR4 levels in hematopoietic stem/progenitor cell (HSPCs) sorted from BM at day 5 after 5FU treatment (n = 3 mice per group). **(F)**: SDF-1α levels in plasma and BM supernatants at day 10 after 5FU treatment (n = 5 mice per group). **(G)**: Representative gelatin zymograms and quantification of total gelatinolytic activity of MMP-9 in BM supernatants of WT or NPY$^{-/-}$ mice (n = 4 per group). **(H and I)**: The (H) mRNA levels in BM and (I) protein levels of HSPC maintenance factors (SDF-1α Kitl, Angpt1 and Vcam1) in BM supernatants of WT or NPY$^{-/-}$ mice (n = 6 mice per group). M: Marker, P: MMP-9 positive control. *, $p < .05$. Data represent three independent experiments. All error bars indicate s.e.m. All expression levels are relative to Gapdh mRNA. Abbreviation: NPY, neuropeptide Y.

peripheral denervation in BM induces declining mobilization in steady state and stress situations [22–24].

Neuropeptide Y (NPY) is secreted from the brain or sympathetic nerves in the autonomic system by various stressors, and has important roles in a variety of physiological processes such as appetite, energy storage, anxiety and pain [25–27]. In bone marrow NPY has been shown to mediate bone, vascular or immune homeostasis through Y receptors expressed in niche cells such as osteoblasts, endothelial and immune cells [28–30]. Moreover, in previous study we revealed that NPY is required for HSPC survival and maintenance of bone marrow function by protecting SNS fibers and bone marrow niche cell survival through TGF-β secreted from macrophages [31, 32]. However, the specific role of NPY in HSPC mobilization has not been fully described.

In this study, we show impaired HSPC mobilization in NPY deficient mice, and that mobilization is enhanced by pharmacological or endogenous NPY increase. The mobilization is induced by reducing HSPC maintenance factors, regulating matrix metalloproteinase-9 (MMP-9) activity through Y1 receptor in osteoblasts. Furthermore, we demonstrate that NPY treatment relieves ovariectomy-induced bone loss in mice through HSPC mobilization, suggesting a functional role of NPY as a regulator of HSPC mobilization and indicating its potential therapeutic use in stem cell based treatments.

Results

Impairment of HSPC Mobilization by NPY Deficiency

To assess the effects of NPY on HSPCs mobilization, we induced mobilization with AMD3100 (5 mg/kg, i.p) or G-CSF (125 μg/kg s.c. for 5 days) in WT or NPY$^{-/-}$ mice. NPY$^{-/-}$ mice showed a reduction in the number of mobilized HSPCs in the peripheral blood (PB) after AMD3100 or G-CSF treatment (Fig. 1A). AMD3100 and G-CSF induce HSPC mobilization by regulating SDF-1α levels [33, 34]. We analyzed SDF-1α levels in plasma and BM supernatants after treatment of AMD3100 or G-CSF. SDF-1α levels increased in plasma and decreased in BM of AMD3100 treated WT mice; G-CSF treated WT mice showed decreased SDF-1α levels in BM. In contrast, NPY$^{-/-}$ mice treated AMD3100 or G-CSF did not show these tendencies (Fig. 1B). Interestingly, NPY$^{-/-}$ mice expressed higher SDF-1α levels in BM compare to WT mice.

Recently 5-fluorouracil (5FU), which induces repopulation of quiescent HSPCs in BM, also was shown to mobilize HSPCs by increasing of expression of fibroblast growth factor receptor 1 (FGFR1) in HSPCs. FGFR1 was required for upregulation of CXCR4 in HSPCs, and 5FU elevated SDF-1α levels in PB [35]. To confirm whether NPY was involved in these processes, WT or NPY$^{-/-}$ mice were treated with 5FU, and expression of FGFR1/SDF-1α/CXCR4 elements was assessed in HSPCs sorted

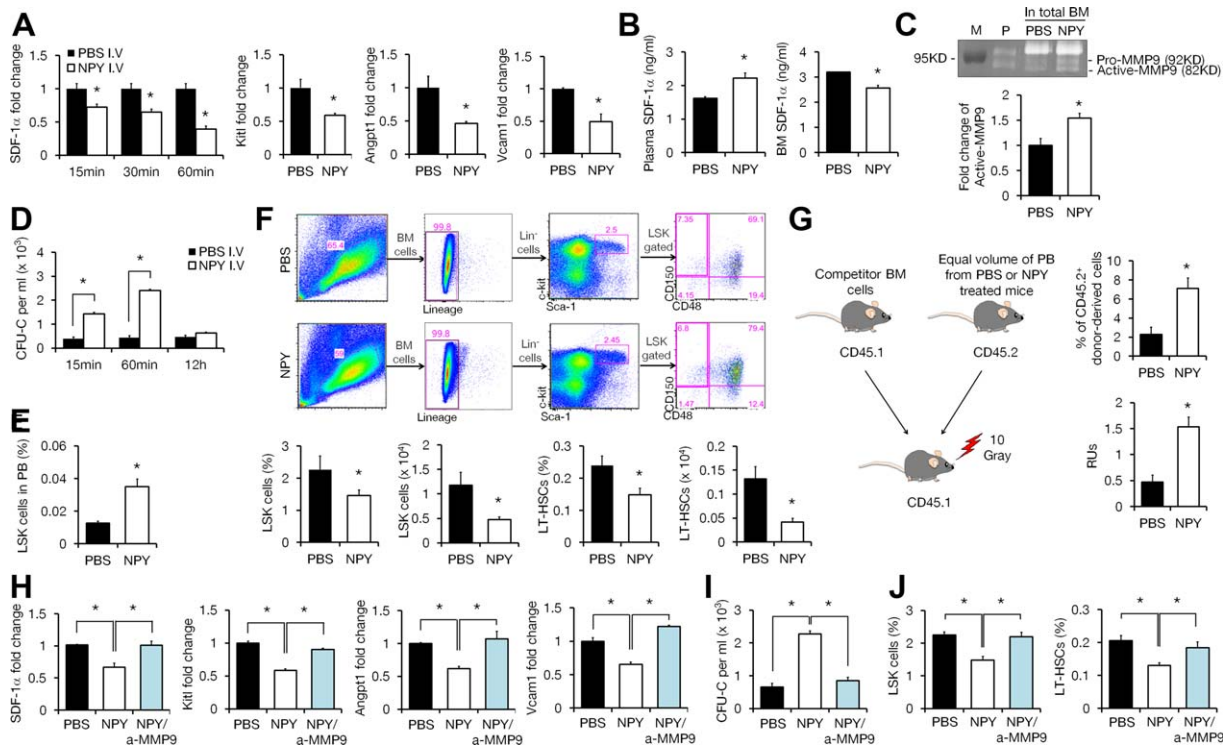

Figure 2. NPY induces hematopoietic stem/progenitor cell (HSPC) mobilization. **(A)**: Expression levels of SDF-1α in the total BM at 15, 30, and 60 minutes and Kitl, Angpt1 and Vcam1 mRNA levels at 60 minutes after PBS or NPY injection ($n = 6$ mice per group, i.v.). **(B)**: SDF-1α levels in plasma and BM supernatants at 60 minutes after NPY treatment ($n = 5$ mice per group). **(C)**: Representative gelatin zymograms and quantification of total gelatinolytic activity of MMP-9 in BM supernatants of PBS or NPY treated mice ($n = 4$ per group). **(D)**: Number of CFU-C in PB at 15 minutes, 60 minutes, and 12 hours after PBS or NPY injection ($n = 6$ mice per group). **(E, F)**: Percentage of (E) Lineage$^-$Sca-1$^+$ckit$^+$ (LSK) cells in PB, and (F) LSK cells and LT-HSCs in BM of PBS or NPY treated mice ($n = 6$ mice per group). **(G)**: Left, experimental design for competitive transplantation. Right, the lethally irradiated CD45.1 recipients were injected with identical volumes of peripheral blood from PBS or NPY treated mice (CD45.2) in competition with identical numbers of CD45.1$^+$ BMNCs. After 16 weeks, the percentage of CD45.2$^+$ donor derived cells and RUs were measured by flow cytometry in PB ($n = 4$ mice per group). **(H)**: Expression levels of HSPC maintenance factors in BM of PBS, NPY, or NPY/MMP-9 neutralizing monoclonal antibody treated mice ($n = 4$ mice per group). **(I, J)**: Number of (I) CFU-C in PB and percentage of (J) LSK cells, LT-HSCs in BM of each group ($n = 4$ mice per group). M: Marker, P: MMP-9 positive control. *, $p < .05$. Data represent three independent experiments. All error bars indicate s.e.m. All expression levels are relative to Gapdh mRNA. See also Supporting Information Figs. S1, S2. Abbreviation: NPY, neuropeptide Y.

from BM. We confirmed the mobilization of HSPCs in PB of WT mice. However NPY$^{-/-}$ mice did not show mobilization (Fig. 1C), although FGFR1 and CXCR4 levels in HSPCs were increased in both WT and NPY$^{-/-}$ mice after 5FU treatment (Fig. 1D, 1E). Because SDF-1α levels did not increase in plasma and also did not decrease in BM of 5FU treated NPY$^{-/-}$ mice (Fig. 1F). These results suggested that impaired HSPC mobilization in NPY$^{-/-}$ mice was associated with increased SDF-1α levels in BM.

Previous study revealed that the degradation of BM SDF-1α by MMP-9 is a vital process in AMD3100 or G-CSF induced HSPC mobilization [36, 37]. These reports encourages that MMP-9 activity may be decreased in BM of NPY$^{-/-}$ mice. We performed the MMP-9 zymography and found reduction of active MMP-9 in BM of NPY$^{-/-}$ mice compared to WT mice despite of AMD3100 or G-CSF treatment (Fig. 1G). Therefore, abnormal increase of BM SDF-1α in NPY$^{-/-}$ mice was due to decrease of MMP-9 activity by NPY deficiency, resulting in impairment of HSPC mobilization.

HSPC maintenance factors such as SDF-1α, Kitl, Angpt1, and Vcam1 [6–9] are important in maintaining the association of HSPCs with niche cells such as osteoblasts and MSCs, and reduction of these factors induces HSPC mobilization [14, 23]. We therefore evaluated the mRNA and protein levels of these factors in total BM of WT and NPY$^{-/-}$ mice. As expected, the expression and protein levels of HSPC maintenance factors was increased in BM of NPY$^{-/-}$ mice, supporting the fact impairment of HSPC egress in NPY$^{-/-}$ mice is related to an increase of HSPC maintenance factors in BM (Fig. 1H, 1I). In previous study, we performed migration assays of HSPCs sorted from BM of WT or NPY$^{-/-}$ mice [31]. The results showed no significant difference between migrated HSPCs of WT and NPY$^{-/-}$ mice with or without SDF-1α suggesting impaired HSPC mobilization of NPY$^{-/-}$ mice was not a cell-autonomous effect. Overall, these results indicated that NPY deficient HSPCs have the normal migration ability, promoting that impaired HSPC mobilization in NPY$^{-/-}$ mice is due to increase of HSPC maintenance factors by reduction of MMP-9 activity in BM.

HSPC Mobilization by NPY

To further investigate a possible role of NPY in mobilization, we injected WT mice with NPY intravenously (i.v.) and collected BM after 15, 30, and 60 minutes. We observed reduction of HSPC maintenance factors at 60 minutes after NPY injection (Fig. 2A). A previous study reported that rapid HSPC

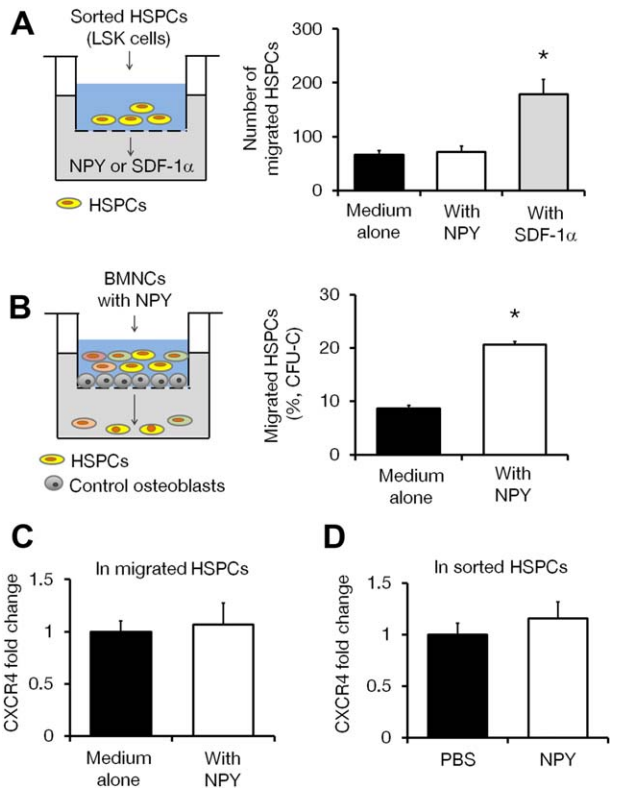

Figure 3. NPY does not have chemotactic ability itself and does not affect CXCR4 levels in HSPCs. **(A)**: Illustration of transwell migration assay and comparison of the migration ability of HSPCs in the presence of NPY ($n = 3$ per group). **(B)**: Illustration of transstromal migration assay and comparison of the migration of HSPCs in the presence of NPY ($n = 3$ per group). **(C, D)**: Expression of CXCR4 (C) in migrated HSPCs of lower chamber ($n = 3$ per group) and (D) in HSPCs sorted from BM of PBS or NPY treated mice (Three experiments in which populations were sorted from 10 mice each). *, $p < .05$. Data represent three independent experiments. All error bars indicate s.e.m. All expression levels are relative to Gapdh mRNA. Abbreviations: HSPCs, hematopoietic stem/progenitor cell; LSK, Lineage$^-$Sca-1$^+$ckit$^+$; NPY, neuropeptide Y.

mobilization involves SDF-1α secretion from the BM into the PB [34], and here we found that NPY treatment also increased SDF-1α levels in plasma and decreased levels in BM after 60 minutes by increasing MMP-9 activity in BM (Fig. 2B, 2C). Reduced HSPC maintenance factors in BM and elevated SDF-1α secretion into PB by NPY treatment induced mobilization of HSPCs subsets such as hematopoietic progenitors, Lineage$^-$Sca-1$^+$ckit$^+$(LSK) cells, and LSKCD48$^-$CD150$^+$ long-term HSCs (LT-HSCs) (Fig. 2D–2F). Conversely, CD45$^-$Lin$^-$CD31$^-$Sca1$^+$CD51$^+$ MSCs, CD45$^-$Lin$^-$CD31$^-$Sca1$^-$CD51$^+$ osteoblasts, and CD3$^+$ T cells were increased in NPY treated mice (Supporting Information Fig. S1A, S1B).

We also performed competitive transplantation studies to reconfirm the effects of NPY on HSPC mobilization. Transplantation of BM from NPY treated mice resulted in a increased percentage of CD45.2$^+$ donor-derived cells and repopulation units (RUs) in PB compared to injection of PB from PBS treated mice, further suggesting the importance of NPY on HSPC mobilization from BM (Fig. 2G). To confirm HSPC mobilization by long-term follow up of NPY treatment, mice received i.p. injection of NPY twice daily for 6 days. The expression of maintenance factors in BM was reduced throughout the treatment period and mobilization of HSPCs was increased in NPY treated mice (Supporting Information Fig. S2A, S2B). Overall, these data indicated that NPY mobilized HSPCs from the BM to the PB via reduction of HSPC maintenance factors in BM.

Finally, to determine whether MMP-9 mediates reduction of HSPC maintenance factors and mobilization by NPY, we injected MMP-9 neutralizing monoclonal antibody (a-MMP9) with NPY. The injection of a-MMP9 caused inhibition of maintenance factors reduction and HSPC mobilization by NPY (Fig. 2H–2J). These findings indicate that increase of MMP-9 activity by NPY reduces expression of HSPC maintenance factors, resulting in HSPC mobilization.

Next, to investigate the possibility of direct HSPC mobilization effects by NPY, we performed in vitro migration assays. Five-thousand HSPCs sorted from BM of WT mice were placed into the top chamber of a 24-well transwell plate, and NPY was included in the media of the lower chamber. The result showed no significant difference between migrated HSPCs with or without NPY (Fig. 3A). These results suggested that NPY did not have chemotactic ability itself. Based on these findings, we performed an in vitro transstromal migration assay. 5×10^4 BMNCs with or without NPY treatment were placed in the top chamber of 24-well transwell plate containing a monolayer of osteoblasts, which are one of the stromal cells expressing HSPC maintenance factors. The migration capacity was measured by CFU-C assays. HSPCs migration to the bottom chamber was increased by NPY treatment (Fig. 3B). We also confirmed CXCR4 levels in migrated HSPCs, and found no significant difference between each group. Similar result was obtained in HSPCs sorted from BM of PBS or NPY treated mice (Fig. 3C, 3D). Therefore, these results indicated that NPY mediated HSPC mobilization by regulating HSPC maintenance factors in niche cells such as osteoblasts.

The Y1 Receptor in HSPC Mobilization

We next attempted to determine whether the Y1 receptor mediated the HSPC mobilization effect of NPY. Y1 antagonist treated mice did not reduce HSPC maintenance factors (Supporting Information Fig. S3A) and resulted in inhibition of NPY-induced HSPC mobilization (Supporting Information Fig. S3B–S3D). We obtained similar results in competitive transplantation experiments (Supporting Information Fig. S3E). In contrast to Y1 antagonist treatment, Y1 agonist treatment induces mobilization of HSPC subsets as the biggest phenotype similar to NPY treatment itself (Supporting Information Fig. S4A–S4E). Taken together, these data revealed that NPY induced HSPC mobilization through the Y1 receptor.

As discussed above, osteoblasts and MSCs are major niche cells maintaining HSPCs by expressing maintenance factors [6–13]. These cells also are known to express Y1 receptors [28, 38]. To reveal whose Y1 receptors mediated HSPC mobilization by NPY, we first treated osteoblasts differentiated from MSCs with NPY for 3 days (Fig. 4A). NPY caused reduced expression of HSPC maintenance factors in these cells, particularly SDF-1α in a concentration dependent manner, but did not have an effect in nondifferentiated MSCs (Fig. 4B, 4C). To confirm this observation further in vivo, we evaluated HSPC maintenance factors in osteoblasts sorted from whole BM of PBS or NPY treated mice (see osteoblasts sorting strategy in Supporting Information Fig. S1A). These factors were decreased in osteoblasts sorted from NPY treated mice (Fig.

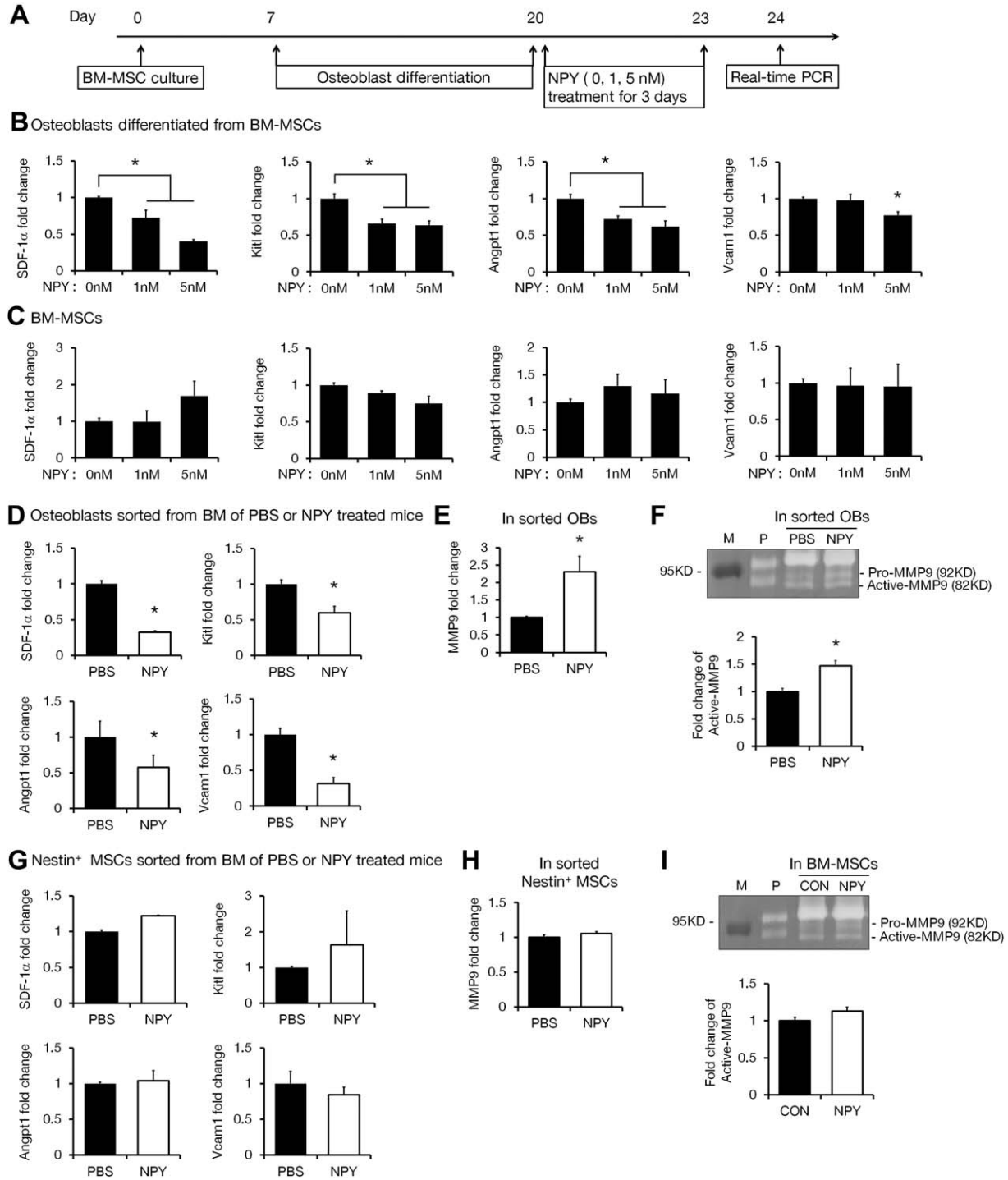

Figure 4. NPY reduces Hematopoietic stem/progenitor cell (HSPC) maintenance factors in osteoblasts by increasing MMP-9 activity. **(A)**: Experimental design to determine the effect of NPY on HSPC maintenance factors (SDF-1α, Kitl, Angpt1, and Vcam1) in osteoblasts or BM-MSCs. **(B, C)**: Expression levels of factors in (B) osteoblasts or (C) BM-MSCs following concentration NPY treatment ($n = 4$–6 per group). **(D, E)**: Expression levels of (D) HSPC maintenance factors and (E) MMP-9 in osteoblasts sorted from BM of PBS or NPY treated mice. **(F)**: Representative gelatin zymograms and quantification of total gelatinolytic activity of MMP-9 in osteoblasts sorted from BM of each groups. **(G, H)**: Expression levels of (G) HSPC maintenance factors and (H) MMP-9 in nestin$^+$ MSCs sorted from BM of each groups. **(I)**: Representative gelatin zymograms and quantification of total gelatinolytic activity of MMP-9 in MSCs cultured from BM with or without NPY treatment ($n = 4$ per group). Three experiments in which osteoblasts or nestin-GFP$^+$ cells were sorted from BM of 10 mice each. M: Marker, P: MMP-9 positive control. *, $p < .05$. Data represent three independent experiments. All error bars indicate s.e.m. All expression levels are relative to Gapdh mRNA. See also Figs. S3–S6. Abbreviation: NPY, neuropeptide Y.

4D). Moreover, osteoblasts sorted from BM of NPY treated mice showed significantly increased MMP-9 expression and activity compare to osteoblasts sorted from BM of PBS treated mice (Fig. 4E, 4F). Nestin$^+$ MSCs are strictly perivascular, exist in central areas of the marrow, and also express HSPC maintenance factors. Reduction of nestin$^+$ MSCs also leads to HSPC mobilization [13, 14]. We therefore sorted nestin$^+$ MSCs from whole BM after PBS or NPY injection in *Nes*-GFP mice [13], and observed no reduction of HSPC maintenance factors and increase of MMP-9 expression (Fig. 4G, 4H). The activity of MMP-9 also did not alter in BM-MSCs with or without NPY treatment (Fig 4I). These results indicated that NPY led to HSPC mobilization by increasing MMP-9 activation in osteoblasts which caused reduction of HSPC maintenance factor expression, especially SDF-1α.

The suppression of osteoblasts induces HSPC mobilization in the endosteal niche [23], and we therefore investigated expression of osteoblast specific genes in vitro and in vivo with or without NPY treatment, and in NPY$^{-/-}$ mice. However, we found no significant differences between these groups (Supporting Information Fig. S5A–S5D), suggesting that HSPC mobilization by NPY occurs by only reducing HSPC maintenance factors expression in endosteal osteoblasts without suppression of osteoblasts.

In Fig. 1G, 1H, we observed increase of HSPC maintenance factors by reduction of MMP-9 activity in whole BM of NPY$^{-/-}$ mice. To confirm whether these observations were derived from osteoblasts, we sorted osteoblasts from whole BM of WT or NPY$^{-/-}$ mice and found similar results (Supporting Information Fig. S6A, S6B). We further investigated that NPY induces HSPC mobilization of NPY$^{-/-}$ mice by regulating maintenance factors in osteoblasts. The results showed that reduction of maintenance factors in osteoblasts and mobilized HSPCs in NPY$^{-/-}$ mice by NPY treatment, indicating NPY restores impaired HSPC mobilization of NPY$^{-/-}$ mice (Supporting Information Fig. S6C, S6D).

We next confirmed whether the Y1 receptor in osteoblasts mediates the HSPC mobilization effect by activating MMP-9. We treated osteoblasts with Y1 antagonist or Y1 agonist in vitro, and found that the Y1 agonist only reduced HSPC maintenance factors (Supporting Information Fig. S7A, S7B). Moreover, MMP-9 activity increased in osteoblasts sorted from Y1 agonist treated mice, but not in Y1 antagonist (Supporting Information Fig. S7C). These results indicated that NPY mediated HSPC egress through the Y1 receptor in osteoblasts by increasing MMP-9 activity.

To more specifically investigate the role of osteoblasts in this phenomenon, col1α1-cre [39] and Y1$^{fl/fl}$ mice [40] were used to conditionally deplete the Y1 receptor in osteoblasts. Y1 receptor mRNA levels were significantly reduced in osteoblasts sorted from col1α1-cre; Y1$^{fl/fl}$ mice (Supporting Information Fig. S7D). We then injected PBS or NPY into these mice. NPY treated col1α1-cre; Y1$^{fl/fl}$ mice did not exhibit a reduction of HSPC maintenance factors and increase of MMP-9 activity in osteoblasts sorted from each mice, resulting in no induction of HSPC mobilization (Fig. 5A–5E). Similar results were obtained in competitive transplantation experiments (Fig. 5F). Y1 receptor deficiency in osteoblasts did not affect the percentage of LSK cells or LT-HSCs in PB and BM (Fig. 5D, 5E; Supporting Information Fig. S10A). These results indicated that Y1 receptor in osteoblasts was not involved in HSPC function itself, promoting that NPY-mediated Y1 receptor stimulation induces MMP-9 activity in osteoblasts and contributes to HSPC mobilization. Next, we performed in vitro transstromal migration assays to reconfirm NPY/Y1 receptor mediated HSPC mobilization in osteoblasts. Importantly, the migration of HSPCs through Y1 receptor depleted osteoblasts was not induced despite NPY treatment (Fig. 5G). Together, these results support the fact that NPY mobilized HSPCs by regulating Y1 receptor in osteoblasts. Furthermore, we confirmed MMP-9 activation in control or Y1 receptor depleted osteoblasts with or without NPY treatment. MMP-9 activation did not increase in NPY treated Y1 receptor depleted osteoblasts (Fig. 5H). Therefore, NPY/Y1 receptor induced reduction of HSPC maintenance factors by increasing MMP-9 activity in osteoblasts, resulting in HSPC mobilization.

The MMP-9 as a Mediator on NPY/Y1 Receptor Induced-HSPC Mobilization

To determine whether forced activation of MMP-9 in Y1 receptor depleted osteoblasts restores NPY-induced HSPC migration, in vitro transstromal migration assays was performed with or without MMP-9. The migration of HSPCs through Y1 receptor depleted osteoblasts was induced by MMP-9 treatment (Fig. 6A). We further confirmed the functional role of MMP-9 on NPY/Y1 receptor induced-HSPC mobilization in vivo. Col1a1-cre; Y1$^{fl/fl}$ mice were injected with MMP-9, and the results showed reduction of HSPC maintenance factors and induction of HSPC mobilization (Fig. 6B–6D). These results suggest that MMP-9 activation by NPY/Y1 receptor stimulation mediates HSPCs mobilization. To more prove whether the MMP-9 in osteoblasts is responsible for the HSPC mobilization by NPY/Y1 receptor stimulation, in vitro transstromal migration assays performed in MMP-9 depleted osteoblasts. The migration of HSPCs through MMP-9 depleted osteoblasts was not induced despite NPY treatment (Fig. 6E), promoting the MMP-9 as a pivotal mediator of the HSPC mobilization by NPY/Y1 receptor.

Macrophages regulating HSPC traffic reside in the perivascular niche as well as in the endosteal niche with osteoblasts. We first confirmed MMP-9 activity of BM- macrophages with or without NPY treatment in vitro and observed no significant difference (Supporting Information Fig. S8A). CD11b$^+$F4/80$^+$Ly6G$^+$ and F4/80$^+$CD115$^+$Gr1$^-$CD169$^+$ macrophages are involved in HSPC traffic in both endosteal and perivascular niches, and loss of these macrophages causes mobilization of HSPCs out of BM into the PB [41, 42]. Previous study reported that αSMA expressing macrophages preserved HSPCs in the perivascular niche, although it was a very small population of the total BM [43]. We therefore examined the numbers of these macrophage subsets in BM of PBS or NPY treated mice, and found no significant change the numbers of any macrophage group with either treatment (Supporting Information Fig. S8B–S8D). These results suggested that the Y1 receptor in macrophages was not associated with NPY-mediated HSPC mobilization. In order to more specifically investigate the question of whether Y1 receptor expression in macrophages regulates HSPC mobilization, Lyz2-cre recombined in the myeloid cell lineage [44] mice were used; reduction of Y1 receptor mRNA levels was observed in macrophages cultured from BM of Lyz2-cre; Y1$^{fl/fl}$ mice (Supporting Information Fig. S8E). Then, control or Lyz2-cre; Y1$^{fl/fl}$ mice received PBS or NPY injections, and we found mobilized

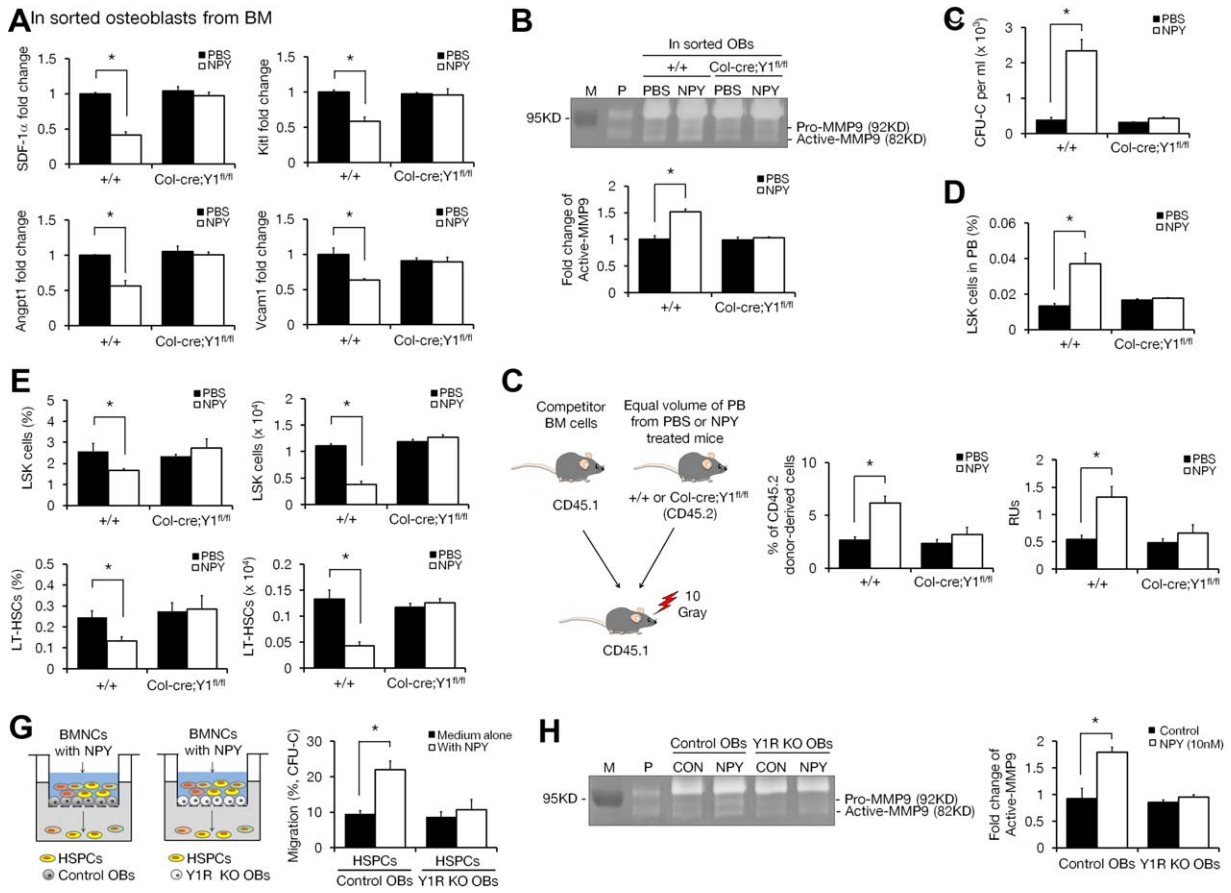

Figure 5. Y1 receptor in osteoblasts is required for HSPC mobilization by NPY. **(A, B)**: Expression levels of (A) HSPC maintenance factors and (B) quantification of total gelatinolytic activity of MMP-9 in osteoblasts sorted from BM of PBS or NPY treated control and col1α1-cre; $Y1^{fl/fl}$ mice (Three experiments in which osteoblasts were sorted from BM of 10 mice each). **(C)**: Number of CFU-C in PB of PBS or NPY treated control and col1α1-cre; $Y1^{fl/fl}$ mice ($n = 5$–6 mice per group, i.v.). **(D, E)**: Percentage of (D) LSK cells in PB, and (E) $Lineage^{-}Sca\text{-}1^{+}ckit^{+}$ (LSK) cells and LT-HSCs in BM of each groups ($n = 4$–6 mice per group). **(F)**: Left, experimental design for competitive transplantation. Right, the lethally irradiated CD45.1 recipients were injected with identical volumes of peripheral blood from PBS or NPY treated control and col1α1-cre; $Y1^{fl/fl}$ mice (CD45.2) in competition with identical numbers of $CD45.1^{+}$ BMNCs. After 16 weeks, the percentage of $CD45.2^{+}$ donor derived cells and RUs were measured by flow cytometry in PB ($n = 4$ mice per group). **(G)**: Migration of HSPCs through control (Control OBs) or Y1 receptor downregulated osteoblasts (Y1R KO OBs) with or without NPY ($n = 3$ per group). **(H)**: Representative gelatin zymograms and quantification of total gelatinolytic activity of MMP-9 in osteoblasts of control and Y1 receptor downregulated with or without NPY treatment ($n = 4$ per group). +/+, wild-type. M: Marker, P: MMP-9 positive control. *, $p < .05$. Data represent three independent experiments. All error bars indicate s.e.m. All expression levels are relative to Gapdh mRNA. See also Supporting Information Figs. S7, S10. Abbreviations: HSPC, hematopoietic stem/progenitor cell; NPY, neuropeptide Y.

HSPCs in the NPY treated animals (Supporting Information Fig. S8F, S8G). Therefore, depletion of Y1 receptor in macrophages did not alter the NPY-mobilization effect, and we therefore conclude that NPY regulates HSPC mobilization through Y1 receptor in osteoblasts by increasing MMP-9 activation in the endosteal niche, but not in macrophages.

HSPC Mobilization by Endogenous NPY

NPY is preferentially released during prolonged and/or intense stress [25]. To determine whether endogenously released NPY from sympathetic nerves induces HSPC mobilization, mice were first restrained in a conical tube for 1 month and exposed to cold water for 2 weeks (Supporting Information Fig. S9A). We found increased NPY levels in the blood of cold stress (CS), but not in restraint/isolation (RI), stress groups compared to no stress (NS) groups. However, NPY levels in BM increased in both groups (Supporting Information Fig. S9B, S9C). Increased NPY levels in BM led to a significant reduction of HSPC maintenance factors by increasing of MMP-9 expression in whole BM and activation in osteoblasts, and it caused induction of HSPC mobilization (Supporting Information Fig. S9D–S9H). We then subjected $NPY^{-/-}$ and col1α1-cre; $Y1^{fl/fl}$ mice to RI stress and CS, and NPY levels were determined in blood and BM of each group (Supporting Information Fig. S9I, S9J). Notably, NPY deficiency or absence of Y1 receptors in osteoblasts did not affect expression of HSPC maintenance factors in BM by stress (Supporting Information Fig. S9K). Moreover, MMP-9 expression in osteoblasts sorted from BM of each group did not increase in $NPY^{-/-}$ and col1α1-cre; $Y1^{fl/fl}$ mice group, and these mice group did not show HSPC mobilization (Supporting Information Figs. S9L–S9N, S10B–S10C). Therefore, these results indicated that the release of endogenous NPY from sympathetic nerves to BM by chronic stress mediated HSPC mobilization through the Y1

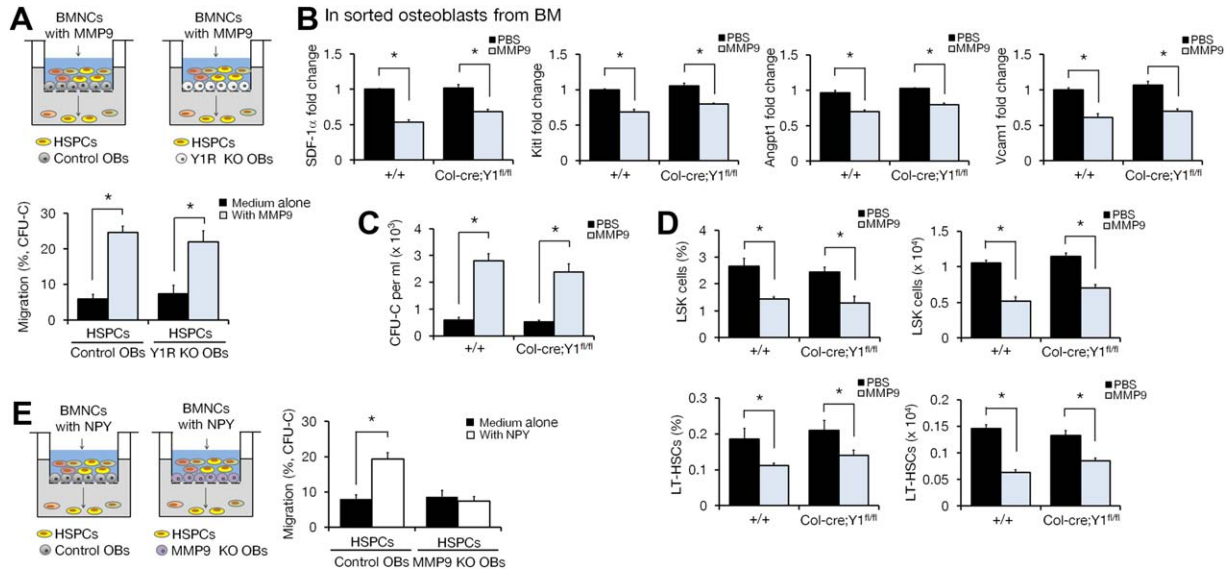

Figure 6. MMP-9 plays as a mediator for NPY/Y1 receptor induced HSPC mobilization. **(A)**: Migration of HSPCs through control or Y1 receptor downregulated with or without MMP-9 ($n = 3$ per group). **(B)**: Expression levels of HSPC maintenance factors in osteoblasts sorted from BM of $+/+$ or col-cre; $Y1^{fl/fl}$ mice with or without MMP-9 treatment (Three experiments in which osteoblasts were sorted from BM of 10 mice each). **(C, D)** The number of (C) CFU-C in PB and (D) the percentage of LSK cells and LT-HSCs in BM of each group ($n = 4$ mice per group). **(E)**: Migration of HSPCs through control or MMP-9 downregulated osteoblasts with or without NPY ($n = 3$ per group). *, $p < .05$. Data represent three independent experiments. All error bars indicate s.e.m. All expression levels are relative to Gapdh mRNA. See also Supporting Information Fig. S8. Abbreviations: HSPC, hematopoietic stem/progenitor cell; NPY, neuropeptide Y.

receptor by regulating MMP-9 activity and HSPC maintenance factors in osteoblasts.

Maintenance of Bone Homeostasis by NPY

Bone homeostasis is required for the balance between bone-forming osteoblasts and bone resorbing osteoclasts [45]. Previous studies demonstrated that AMD3100-induced mobilization of HSPCs reduces bone loss in an ovariectomy-induced osteoporosis mice model by decreasing the number of osteoclasts differentiated from BM-derived HSPCs [46]. Moreover, recruitment of osteoclast precursors from BM to blood also relieves bone loss in ovariectomized mice through reduction of osteoclast accumulation onto bone surfaces [47]. We therefore hypothesized that the mobilization of HSPCs by NPY decreases the number of osteoclasts in BM since osteoclasts are differentiated from HSPCs [48]. To investigate this hypothesis, twelve week-old female mice underwent either sham surgery or ovariectomy. One week after surgery, the sham and ovariectomized mice received an i.p. injection with PBS or NPY for 21 days. At the end of treatment, we checked body and uterine weight in order to ensure success of the ovariectomy procedure and the effects of treatment; the results showed increased body weight and reduced uterine weight in ovariectomized mice compared to sham-operated mice (Supporting Information Fig. S11A). HSPC maintenance factor expression levels also significantly increased in the BM of ovariectomized mice, indicating that BM of ovariectomized mice contained more HSPCs than that of sham-operated mice (Supporting Information Fig. S11B, S11C). However, this did not affect the number of hematopoietic progenitors in blood (Supporting Information Fig. S11D). Also, increased SDF-1α expression in BM from ovariectomized mice was significantly reduced by NPY treatment, and HSPCs were mobilized (Supporting Information Fig. S11E–S11G).

Next, the femora of either sham or ovariectomized mice, which were injected with NPY, were analyzed by μCT. NPY treatment significantly prevented bone density loss after ovariectomy, and similar effects were observed under control (sham non-overiectomized mice) conditions (Supporting Information Fig. S11H, Supporting Information Movie S1–S4). Moreover, the number and size of $TRAP^+$ active osteoclasts was decreased in the trabecular region in the NPY treated ovariectomized mice (Supporting Information Fig. S11I). To investigate the possibility of osteoclast precursors mobilization by NPY, $CD11b^+$ osteoclast precursors/monocytoid cells were measured in PB and BM according to a previous studies [47, 49]. The results showed that ovariectomized-mice did not induce mobilization of $CD11b^+$ cells by NPY treatment. However, $CD11b^+$ cells in BM were reduced in NPY injected ovariectomized-mice (Supporting Information Fig. S11J). Next, we investigated whether NPY induced HSPC differentiation into mature functional osteoclasts to clarify whether NPY leads to HSPC mobilization to blood or reduced differentiation into mature osteoclasts. The results showed that NPY did not have an effect on mature osteoclast specific genes expression (Supporting Information Fig. S11K). Therefore, these findings indicated that the decreased number of osteoclasts in ovariectomized-mice was caused by NPY mediated HSPC mobilization. Finally, we determined the effects of NPY treatment on osteoblast activity because our data indicated an increased percentage of osteoblasts in BM of NPY treated C57BL/6 mice (see Supporting Information Fig. S1A). We performed H&E staining in order to determine the effect of NPY on the number of osteoblasts. These findings revealed that osteoblast numbers were significantly increased in the NPY treated

groups (Supporting Information Fig. S11L). Overall, these results suggested that NPY relieved ovariectomy induced bone loss by reducing the number of osteoclasts through HSPCs mobilization, as well as by increasing the number of osteoblasts.

Discussion

Many reports indicate a functional link between the nervous system and HSPC mobilization. Neurotransmitters secreted from sympathetic nerves play a key role in this process [17–21]. NPY is one of the most abundant and widely secreted peptides from the brain or sympathetic nerves [25]. However, the specific function of NPY on HSPC mobilization has not been fully characterized. Here we demonstrate the unknown role of NPY as a new regulator of HSPC mobilization. NPY deficient mice showed impairment of HSPC mobilization with AMD3100 or G-CSF. These agents induced HSPC egress by increasing SDF-1α levels in blood and/or by reducing levels in BM of WT mice, but not in NPY$^{-/-}$ mice. Similar results were observed in 5FU treated NPY$^{-/-}$ mice. 5FU induced HSPC mobilization by increasing FGFR1 and CXCR4 expression in HSPCs, as well as SDF-1α levels in PB [35]. Although FGFR1/CXCR4 was upregulated in HSPCs of NPY$^{-/-}$ mice, SDF-1α levels did not increase in the PB. Rather, NPY$^{-/-}$ mice showed increased expression of SDF-1α and HSPC maintenance factors in BM. Taken together, these results suggested that impairment of HSPC egress in NPY$^{-/-}$ mice is associated with an increase of SDF-1α and HSPC maintenance factors in BM. The decrease of BM SDF-1α by AMD3100 or G-CSF is related to the enhanced activation of MMP-9. Activation of MMP-9 in response to G-CSF-induced mobilization is well known, and was also documented following AMD3100 administration [36, 37]. Our results showed that reduction of MMP-9 activation in BM of NPY$^{-/-}$ mice led to increased BM SDF-1α resulting in defected HSPC egress (Supporting Information Fig. S12A). Decrease of MMP-9 activity by NPY deficiency is supported by the fact that MMP family members such as MMP-3 are activated by NPY [50].

NPY administration/increase led to HSPC decrease in BM due to mobilization of HSPCs into blood through the Y1 receptor in osteoblasts, and by reducing HSPC maintenance factors in BM. Recent studies have demonstrated that conditional deletion of Kitl from osteoblasts has no effect on HSPC egress, and also that Angpt1 or Vcam1 were not involve in HSPC mobilization from the BM. To the contrary, large changes of SDF-1α levels in osteoblasts regulate HSPC mobilization [51, 52]. In our study, NPY induced HSPC mobilization by reducing expression of SDF-1α in BM and increasing levels in blood through the MMP-9 activation in osteoblasts (Supporting Information Fig. S12B), and long period of NPY treatment also mobilized HSPCs into blood consistently. Of note, the increase of HSPCs mobilization following chronic administration of NPY is not higher than a single treatment. This result may indicate that HSPCs mobilization reaches a new equilibrium state. To investigate the role of NPY as a chemotactic factor, we performed migration assay and found that NPY did not have chemotactic ability of NPY itself. Also, although unexpected, NPY injection resulted in increased MSC, osteoblast and T cell contents in BM. We have confirmed decrease of MSCs and no significant difference of osteoblast in BM of NPY$^{-/-}$ mice in previous our study [31]. In this study, we also confirmed that expression of osteoblast specific genes was not altered in BM of NPY$^{-/-}$ mice. These results suggest that NPY may be involved in MSCs survival but not in differentiation to osteoblasts. The increase of osteoblasts and T cells by NPY is probably associated with direct NPY-stimulated proliferation of these cells, consistent with previous findings [30, 38].

Many studies have shown the importance of vascular niche [3, 13, 14]. We confirmed that NPY did not affect vascular niche cells such as nestin^{+} cells or macrophages inducing HSPC mobilization in vascular niche. Although NPY did not affect HSPC mobilization in this niche, previous our study revealed that NPY regulates HSPC survival within vascular niche by preventing SNS fibers and vascular niche cells [31, 32]. NPY treatment prevented bone loss in a mouse model of osteoporosis through osteoclast reduction by HSPC mobilization in endosteal niche, as well as by an increase of osteoblasts, suggesting that NPY can regulate the molecular interaction between osteoblasts and osteoclasts (Supporting Information Fig. S12C). In addition, chronic stress stimulated the release of NPY from sympathetic nerves, and increased NPY levels in plasma or BM leading to HSPC mobilization through the Y1 receptor in osteoblasts. This conclusion indicates that increased neurotransmitter release from SNS stimulation after external stressors induces mobilization of bone marrow cells, similar to previous results [17].

Recently, regulation of HSPC mobilization has been considered as a treatment standard for a variety of genetic disorders of hematopoiesis [53, 54]. In addition, HSPC mobilization from BM into blood has been used for repairing damage to different tissue and stem cell transplantation [16, 55]. In particular, isolation of circulating HSPCs from the blood has some advantages compared to traditional bone marrow transplantation, such as more rapid engraftment, less pain, lower risk, and fewer technical difficulties compared to harvesting from the BM [56]. Due to these clinical benefits, understanding the involvement of the nervous system as a mediator of HSPC mobilization is important for future clinical applications.

In our study, NPY injection induced rapid HSPC mobilization rapidly showing the highest mobilization into PB within 1 hour after injection. These results suggest that it could be an effective mobilization-inducing agent. Further, our findings provide a potential clinical utility of NPY as a therapeutic agent for bone loss, as well as a potential therapeutic approach for HSPC mobilization. Further studies investigating the functional role of NPY on HSPC mobilization under pathological conditions may provide more effective information on the mechanisms of interaction with the nervous system and HSPC mobilization.

Conclusion

Neurotransmitters released from the SNS regulate HSPC mobilization. NPY is one of the most abundant and widely secreted peptides from the brain or sympathetic nerves, and it has been implicated in a variety of physiological actions. However, the specific function of NPY in the HSPC mobilization has not been fully characterized. Our results suggest that NPY induces HSPC mobilization through Y1 receptor in osteoblasts by

activating MMP-9. Moreover, NPY-induced HSPC mobilization improves bone loss of ovariectomized mice, suggesting new role of NPY as a mediator of HSPC mobilization and potential therapeutic agent for bone loss.

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 [13] have been described previously. $NPY^{-/-}$ mice and control mice were maintained on a 129S1/SvImJ background. Lyz2-cre [44] (The Jackson Laboratory), 2.3-kB col1α1-cre [39], and Y1 flox/flox mice [40] were used to delete the Y1 receptor in osteoblasts and macrophages specifically. The block randomization method was used to allocate the animals to experimental groups. To eliminate bias, investigators were blinded during data collection and data analysis. Mice were housed at a 12 hour 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 HSPC mobilization, control or $NPY^{-/-}$ mice were injected with AMD3100 (5 mg/kg, i.p), G-CSF (125 μg/kg s.c. for 5 days) or 5FU (Sigma; 250 mg per kg body weight, i.v.) under isoflurane anesthesia. To investigate the role of the NPY or Y1 receptor in HSPC mobilization, 5 nM NPY (Bachem, H-6375), 5 nM Y1 agonist [Leu31, Pro34] (Bachem, E-3302), and 5 nM Y1 antagonist BIBP3226 (Bachem, E-3620) were applied to osteoblasts or MSCs in vitro. Three days later, osteoblasts or MSCs were collected for RNA extraction. For in vivo experiment, mice were injected with NPY, Y1 agonist, Y1 antagonist (50 μg per kg body weight, i.v.), MMP-9 neutralizing monoclonal antibody (Millipore, IM09L. 3 mg per kg body weight, i.v.) or recombinant MMP-9 (R&D system, 909-MM-010. 50 μg per kg body weight, i.v.). After 1 hour, the BM was harvested and blood was analyzed by CFU-C assay as described [57].

Bone Marrow Transplantation

The lethally irradiated CD45.1 recipients (BoyJ, total 10 Gy dosage of two split doses) were injected with identical volumes of PB from 6- to 8-week-old mice (CD45.2 donor) in competition with identical numbers of $CD45.1^+$ BMNCs (competitor). Sixteen weeks after transplantation, we quantified the percentage of $CD45.2^+$ cells in the blood of the recipient mice. RUs were calculated according to a previously published method [58].

Flow Cytometry

The BM was flushed from the tibiae and femurs of each mouse. Red blood cells (RBCs) were lysed once for 5 minutes at 4 °C in 0.15 M NH_4Cl (STEMCELL Technologies), washed once with PBS (Gibco), and counted using a hemocytometer. For HSPC, 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 antibiotin (Miltenyi Biotec). For staining of HSPCs, 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 Science. For MSCs and osteoblasts staining, the chopped bone fragments and BM were digested with collagenase type I(3mg/ml; Sigma), and Lin^- cells were stained with APC-Cy7-CD45 (557659), APC-CD31 (551262), Pecy7-Sca1, PE-CD51 (551187), all from BD Science. Cells were further stained with streptavidin-pacific blue (PB) (Invitrogen, S11222). The APC-Cy7-CD45, APC-CD31, PE-Ter119 (BD Science, 553673), Nestin (Abcam, ab6142) and secondary Alexa Fluor 488 (Life technologies, a11029) were used to stain nestin-positive cells after the BM was digested with collagenase type IV (0.2 mg/ml; Sigma). LSK cells, Nestin-positive cells and osteoblasts were sorted. Macrophages subsets, T cells, B cells and oseteoclast precursors/monocytoid cells were detected by staining with antibodies to PE-CD11b (BD science, 557397), FITC-Ly6G (BD Science, 551160), APC-F4/80 (AbD Serotec, MCA497), APCcy7-Gr1 (BD science, 557661), FITC-CD169 (AbD serotec, MCA884F), PE-CD115 (eBioscience, 12-1152), αSMA (Abcam, ab66133), PE-CD3 (BD Science, 555275) and PB-B220 (BD Science, 558108). Data were collected on a BD LSRII system and AriaIII (BD Science) and analyzed using FlowJo software (Tress Star).

Chronic Stress

Mice were exposed to restraint/isolation (RI) stress or cold stress (CS). To achieve RI stress, mice were restrained in a conical tube for 1 month (6 hours/day for 6 day/week). For CS, we placed mice in 0.5 cm ice-cold water for 1 hour per day for 14 day as described [27]. All stress tests were performed daily between 9:00 and 11:00 a.m. We returned stressed mice to their home cages with free access to food and water, and they showed no signs of difficulty in grooming or eating. Nonstressed control mice were housed with same-sex littermates ($n = 3$–5 per cage) and were transported to/from testing rooms but were not otherwise handled. After 14 days or 1 month, plasma and BM were harvested for measurement of the NPY levels (Phoenix Pharmaceuticals Inc).

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 or two way analysis of variance (ANOVA) was used, followed by Tukey's HSD test. All statistical analyses were performed using SPSS statistical software. $p < .05$ was considered to be significant.

Acknowledgments

This work was supported by a grant of the Korea Health Technology R&D Project (HI14C1636) through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea and the Bio & Medical Technology Development Program (2011-0019356) of the National Research Foundation (NRF) of Korea funded by the Ministry of Science, ICT & Future Planning, Republic of Korea. We thank to Dr. Grigori N. Enikolopov generated and provided *Nes*-GFP mice.

Author Contributions

M.H.P. and J.K.L. Acquisition of data, Analysis and interpretation of data, Drafting the article; N.K., Acquisition of data, Analysis of data; W.M., Interpretation of data; J.E.L., Analysis of data; K.T.K., Interpretation of data; H.A., Contributed reagents (mice); H.H., Contributed reagents (mice); E.H.S., Interpretation of data; revising the article; H.K.J., Acquisition of data; Analysis and interpretation of data; revising the article; J.S.B., Conception and design; Acquisition of data; Analysis and interpretation of data; Drafting or revising the article.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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