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Neuropeptide Y and Neuropeptide Y Y5 Receptor Interaction Restores Impaired Growth Potential of Aging Bone Marrow Stromal Cells

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Abstract

Physiological aging impairs the proliferative potential of bone marrow–derived stromal cells. We report here the pro-proliferative response and improved growth characteristics of the aging bone marrow cells subsequent to neuropeptide Y (NPY)/neuropeptide Y Y5 receptor (NPY Y5R) ligand–receptor interaction. Bone marrow cells were isolated from neonatal (2–3 weeks), young (8–12 weeks), and old (24–28 months) rats on the basis of their preferential adherence to plastic surface. After culturing the cells at initial seeding density of 1×10^4 cells/cm^2, we found that the proliferation potential of bone marrow cells declined with age. Real-time polymerase chain reaction (PCR) and Western blotting showed that bone marrow cells in different age groups constitutively expressed NPY and NPY receptor subtypes (Y1R, Y2R, and Y5R). However, NPY and Y5R expression increased by more than 130-fold and decreased by 28-fold, respectively, in old bone marrow cells as compared to young bone marrow cells. NPY (10 nM) stimulated the proliferation of all bone marrow cells age groups, and their proliferation was blocked by Y5R antagonist. However, the pro-proliferative effect of NPY on old bone marrow cells was weaker than other cell groups due to lower Y5R expression. Y5R gene transfection of old bone marrow cells with subsequent $NPY_{3–36}$ (10 nM) treatment significantly increased proliferation of old bone marrow cells (>56%) as compared to green fluorescence protein–transfected control old bone marrow cells. Stimulation of old bone marrow cells by NPY treatment rejuvenated the growth characteristics of aging bone marrow cells as a result of Y5R overexpression.

Introduction

Bone marrow stroma constitutes a heterogeneous cell population derived from the non-blood-forming fraction of the bone marrow cells that are purified as preferentially plastic-adherent fibroblastic cells. The mesenchymal stem cells (MSCs) resident in the adult bone marrow fraction self-renew and show multilineage differentiation potential to adopt osteogenic, chondrogenic, and adipogenic phenotypes.[1–3] Notwithstanding the controversies about their capacity to adopt a cardiac phenotype,[4–7] bone marrow stromal cells (BMCs) have progressed to clinical use for myocardial regeneration.[8,9] However, observations of diminished cardiac reparability[10] and loss of proliferative capacity with physiological aging[11–13] have raised questions about the autologous use of bone marrow–derived cells in elderly patients. Different strategies have been adopted to overcome aging-associated senescence of stem cells, including transduction of the catalytic subunit of telomerase,[14] treatment with Notch-1–specific antibody,[15] use of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitor,[16] and Wnt inhibitor treatment,[17] albeit with little progress. Given that neuropeptide Y (NPY) is mitogenic for several cell types, including smooth muscle cells[18] and endothelial cells,[19,20] via interaction with its specific receptors, the present study was designed to show the effectiveness of stimulation with NPY to overcome the age-related senescence of BMCs and restore their proliferative activity.

NPY is a highly conserved 36-amino-acid pancreatic polypeptide with wide distribution in both central and peripheral nervous systems.[21] It is involved in neuroendocrine mechanisms, cognitive functions, eating behavior, and cardiovascular activity.[22] The primary receptors specific for NPY have wide distribution in the body tissues and bind with substituted or truncated NPY peptides.[22–24] At least six distinct primary NPY receptors, namely YR1 to YR6, have been identified as members of the G protein–coupled receptor family. Interaction of NPY with its Y1 receptor (Y1R)

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and Y5 receptor (Y5R) activates the extracellular signal–regulated kinases (Erk), which are the key intracellular transducers of mitogenic stimulus implicated in the signaling pathway leading to cell proliferation.[25,26] Similarly, NPY/Y1R ligand–receptor interaction has been implicated in the maintenance of putative (undifferentiated) and mature mesenchymal progenitor cell populations[25]; however, it remains unclear whether NPY and its receptor system are associated with the proliferation of BMCs. We observed differential expression pattern of NPY ligand and Y5R in BMCs derived from aging rats (^{Old}BMCs) as compared to BMCs from young rats (^{Yng}BMCs) and neonatal rats (^{Neo}BMCs). We hypothesized that NPY/Y5R ligand–receptor interaction can be modulated to correct physiological aging associated cellular senescence.

Materials and Methods

Purification and expansion of BMCs

The present study conformed to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1985) and protocol approved by the Institutional Animal Care and Use Committee, University of Cincinnati.

For purification and expansion of BMCs, bone marrow was obtained from Fischer 344 rats of different age groups; neonatal (3 weeks), young (8–12 weeks), and old (24–28 months) as described earlier.[27] The cells were cultured in low glucose Dulbecco modified Eagle medium (DMEM; Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT). The adherent, spindle-shaped cell population was expanded for further use during *in vitro* and *in vivo* studies. No more than five passages were allowed for the purified BMCs for propagation *in vitro* before use in the experiments. The cells were assessed for surface marker expression by flow cytometry.[27]

Growth curves of different age-derived BMCs

Cells were seeded at a density of 2×10^4 cells/well (1×10^4 cells/cm^2) in 1.0 mL of MSC complete medium in each well of 24-multiwell plates and cultured at 37°C in a 5% CO_2 incubator. The cells were harvested at stipulated time points of 24 hr, 72 hr, and 6–10 days for counting using a hemocytometer. Doubling time (Dt) was calculated using the following relation; $(t2 - t1)\ \log2/(\log N2 - \log N1)$, where N1 is the number of cells at 24 hr after seeding, t1 is the time of harvesting cells at 24 hr after seeding, N2 is the number of cells at 72 hr after seeding, and t2 is the time of harvesting cells at 72 hr after seeding.[28] The saturation density was expressed as the most number of cells counted in this experiment.

Colony formation

Cells were seeded at a density of 200 cells/6-cm diameter dish (10 cells/cm^2) and were cultured for 14-days, fixed with methanol (Fisher Scientific, Pittsburgh, PA), and stained with Giemsa solution (EM Science, Gibbstown, NJ). The colonies were classified as colonies containing 10–50 cells, 51–100 cells, or more than 100 cells, and their number was counted per dish at 100× magnification using a BX-41 Olympus microscope (Olympus, Tokyo, Japan).

Cell proliferation studies

Cells were seeded at a density of 5×10^3 cells/cm^2 in low-glucose DMEM supplemented with 10% FBS followed by starvation in low-glucose DMEM containing 0.5% FBS. After 24 hr, the culture medium was replaced by low-glucose DMEM containing 0.5% FBS and 10 nM NPY (Sigma-Aldrich, MO) in the presence or absence of specific Y5R blocker. After their respective treatments, the cells were cultured for 10 days. At the end of the stipulated time, the cells were fixed with 4% paraformaldehyde (EM Science, Gibbstown, NJ). The cells were later stained with 0.1% crystal violet solution (Fisher Scientific, Pittsburgh, PA) and counted or quantified spectrophotometrically (A = 570 nm).

Transfection of Y5R into ^{Old}BMCs

^{Old}BMCs were seeded at density of 2×10^5 cells/well (six-well plates) in low-glucose DMEM supplemented with 10% FBS and cultured for 16 hr before transfection with Y5R plasmid DNA. For transfection, the cells were incubated with 0.8 mL/well of low-glucose DMEM plus 0.5% FBS including 2 μg of human Y5R plasmid (gift from Dr. Sheriff, University of Cincinnati) or green fluorescent protein (GFP) plasmid as a control (Clontech Labs, Mountain View, CA) and 7.5 μL of lipofectamine-2000 (Invitrogen, Carlsbad, CA) for 3 hr, followed by dilution with 1.2 mL/well of low-glucose DMEM supplemented with 10% FBS per well. The expression level of human Y5R in the transfected ^{Old}BMCs was determined 3 days after transfection, and the cells were then used in proliferation assay and analysis of Erk1/2 signaling pathway.

Real-time polymerase chain reaction

Quantification of NPY, Y1R, Y2R, and Y5R gene expression in different treatment groups of cells was performed by real-time polymerase chain reaction (PCR) using iQ SYBR-Green Supermix (BioRad Lab, Berkeley, CA) in a Bio-Rad iQ5 optical module.[29] The primary curve method was used to calculate threshold cycle (C_t), which was defined as the cycle at which the fluorescence level would reach a predetermined threshold.[29] Specific primers for NPY, Y1R, Y2R, and Y5R were purchased from SuperArray Bioscience Corp. (Frederick, MD).

Western blot

For analysis of NPY peptide and Y1R, Y2R, and Y5R expression, the cells were washed with ice-cold phosphate-buffered saline (PBS) and harvested with 50–80 μL of ice-cold lysis buffer (50 mM HEPES, 0.1 M NaCl, 5 mM ethylenediaminetetraacetic acid [EDTA], 4.5 mg/mL sodium pyrophosphate, 2.0 mg/mL sodium fluoride [NaF], 0.18 mg/mL sodium orthovanadate, 0.1% (wt/vol) Triton X-100, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride [PMSF]). The cells were scraped into chilled 1.5-mL tubes and incubated on ice for 30 min. For analysis of phosphorylation of Erk1/2 in Y5R-transfected ^{Old}BMCs, Y5R- and GFP- (control) transfected ^{Old}BMCs were incubated in the presence or absence of Y5R blocker (0.25 μM CGP71683A [CGP]; Tocris Biosciences, Ellisville, MO) or Erk1/2 blocker (50 μM PD9859; Santa Cruz Biotechnology Inc., CA) for 30 min before stimulation with 10 nM NPY_{3-36} for 30 min.

After their respective treatments, the cells were rinsed with ice-cold PBS and harvested with 50 μL of ice-cold lysis buffer. The cells were suspended in chilled 1.5-mL tubes and incubated on ice for 30 min at 4°C. After centrifugation (14,000×g for 10 min at 4°C), the supernatant was removed. The cell lysate samples (30–50 μg/lane) were separated on precast 4–12% (wt/vol) continuous gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Invitrogen, Carlsbad, CA) and transferred to polyvinylidene fluoride (PVDF) paper (Bio-Rad, Berkeley, CA), as described earlier.[30] The membrane was washed with Tris-buffered saline (TBS)-T buffer (TBS containing 0.1% Tween-20; Sigma-Aldrich, MO) for 5 min and then for 1 hr with blocking buffer (TBS-T buffer containing 5% nonfat dried milk; Bio-Rad, Berkeley, CA). Primary antibodies were diluted by blocking buffer and incubated with the blot for overnight at 4°C. The antibodies used in this study are shown Table 1. The blot was washed three times for 5 min each using TBS-T buffer and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (diluted in blocking buffer) for 1 hr at room temperature. The blot was washed three times for 5 min each with TBS-T buffer and visualized with the ECL™ Western Blotting Analysis System (GE Healthcare, UK) and X-ray film (Deville, Scientific Inc, NJ). The strength of the signals on the blots was quantified by densitometry using AlphaEaseFC (Version 5.0.2, Alpha Innotech Corp., CA) computer software.

Statistical analysis

Data were presented as mean ± standard error of the mean (SEM). Group comparisons for the difference in mean responses were made by two-tailed Student t-tests. Statistical significance was defined as $p \leq 0.05$. Because of the exploratory nature of this study, no adjustments were made for multiple testing.

Results

Physiological aging impairs proliferation of BMCs

The BMCs from the three age groups were rich in CD29 and CD90 expressing cell populations (Fig. 1a). Proliferation of BMCs increased in all age groups at 1–3 days after cell seeding and then gradually increased in ^{Neo}BMCs, and ^{Yng}BMCs (Fig. 1b). ^{Old}BMC proliferation was very slow and did not show any significant increase in cell number. Estimation of the Dt at 1–3 days and 3–6 days after cell seeding showed that Dt was shortest in ^{Neo}BMCs (33 ± 3.3 hr) at 1–3 days after cell seeding (Fig. 1c). Similarly, Dt determination at 1–3 days showed that ^{Old}BMCs grew slower (58.9 ± 8.9 hr) than ^{Yng}BMCs (42 ± 4.9 hr) (Fig. 1c). This difference became even more evident for Dt at 3–6 days, showing that ^{Old}BMCs grew slower (302.6 ± 35.3 hr) than both ^{Neo}BMCs (135.5 ± 9.9 hr) and ^{Yng}BMCs (195.1 ± 26.3 hr). Saturation density (cells/cm^2) declined with age and was observed as $5.5 \pm 0.07 \times 10^4$ in ^{Neo}BMCs, $3.5 \pm 0.1 \times 10^4$ in ^{Yng}BMCs as compared to $2 \pm 0.1 \times 10^4$ in ^{Old}BMCs (Fig. 1d).

Aging and colonogenicity of BMCs

Colony formation is critically dependent on attachment frequency, the ability of single cells to divide after attachment and growth speed during incubation period.[31] The colonogenicity of BMCs seeded at a density of 10 cells/cm^2 and cultured for 14 days showed that the number of colonies and the number of cells constituting individual colony diminished with aging. Consequently, ^{Old}BMCs showed decreased colonogenicity as compared to both ^{Neo}BMCs and ^{Yng}BMCs (Fig. 2).

NPY ligand–receptor system in different age-derived BMCs

Real time-PCR analysis showed that NPY expression in ^{old}BMCs was 69- and 130-fold higher as compared to ^{Neo}BMCs and ^{Yng}BMCs, respectively (Fig. 3a). Whereas Y1R showed an age-related upregulation (Fig. 3b), Y2R gene was found to be downregulated (Fig. 3c). Similarly, Y5R gene expression increased in ^{Yng}BMCs as compared to ^{Neo}BMCs but showed 28- and 130-fold decline in ^{Old}BMCs as compared to ^{Yng}BMCs and ^{Neo}BMCs (Fig. 3d). NPY and Y1R protein expression also increased with age (Fig. 3e–f). On the other hand, Y2R protein expression decreased with age (Fig. 3g). Y5R expression decreased in ^{Neo}BMCs and ^{Old}BMCs as compared to ^{Yng}BMCs (Fig. 3h).

NPY and BMCs proliferation

Treatment with 10 nM NPY stimulated BMCs proliferation from all the three age groups of animals, but the pro-proliferative effect of NPY was less marked on the ^{Old}BMCs than ^{Neo}BMCs and ^{Yng}BMCs (Fig. 4a). Although several investigators have demonstrated a role for Y1R, Y2R, and Y5R in cell proliferation,[20] we focused on NPY/Y5R ligand–receptor interaction and examined the effect of NPY on the

Table 1. Antibodies Used in Western Blot Studies

Name	*Molecular weight*	*Source*	*Antibody dilutions*	*Company*	*Catalog number*
NPY	4.27 kDa	Rabbit	1/2000	Santa Cruz Biotech, Inc.	sc-28943
Y1R	38.76 Da	Mouse	1/2000	Abnova Corporation	H00004886-M03
Y2R	42.6 kDa	Goat	1/2000	Novus Biologicals, Inc	NB100-57847
Y5R	50.7 kDa	Goat	1/2000	Novus Biologicals, Inc	NB100-1538
Phospho-p44/42 MAP kinase (Thr202/Thy204) [pERK1/2]	42, 44 kDa	Rabbit	1/1000	Cell Signaling Technology	#9101
p44/p42 MAP kinase	42, 44 kDa	Rabbit	1/1000	Cell Signaling Technology	#9102
Actin (I-19)	43 kDa	Goat	1/10000	Santa Cruz Biotech, Inc.	sc-1616

NPY, Neuropeptide Y; Y1R, NPY Y1 receptor; MAP kinase, mitogen-activated protein kinase.

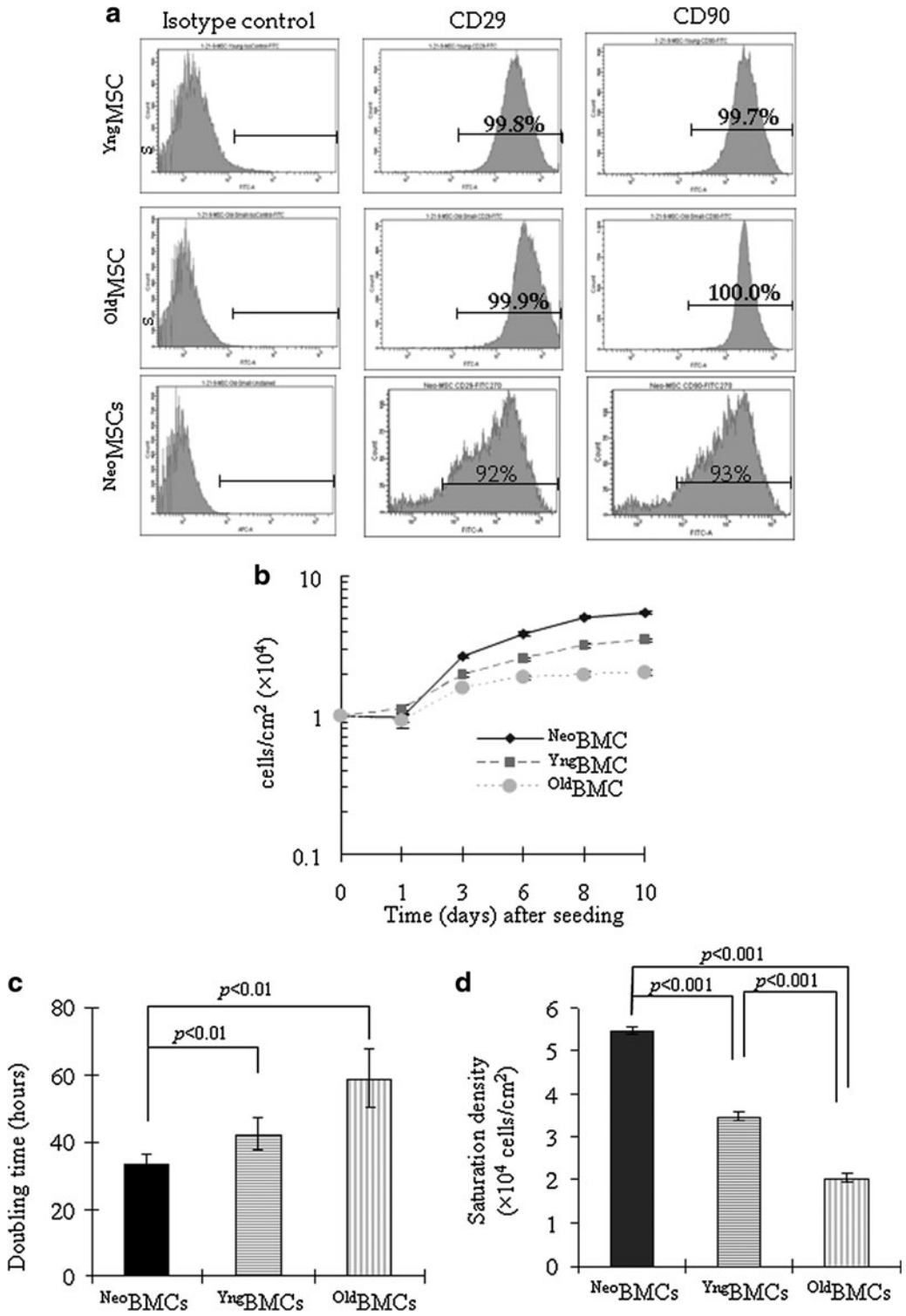

FIG. 1. Surface marker expression and determination of the effect of age on the proliferation potential of bone marrow stromal cells (BMCs). (**a**) Histograms showing that all the BMCs isolated from all three age groups were rich in CD90- and CD29- expressing cell populations. The cells were later seeded at a density of 1×10^4 cells/cm^2 and cultured with low-glucose Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) for cell proliferation studies. Cells were counted on 1, 3 6, 8, and 10 days after cell seeding. (**b**) Growth curves of ^{Neo}BMCs, ^{Yng}BMCs, and ^{Old}BMCs. After cell seeding, cell proliferation increased in all age groups between days 1 and 3; subsequently it gradually increased only in ^{Neo}BMCs, and ^{Yng}BMCs between days 3 and 10 of observation. ^{Old}BMCs did not show any significant increase in cell number. (**c**) The doubling time (Dt) of different age-derived BMCs. ^{Neo}BMCs had the shortest Dt (33 ± 3.3 hr) as compared to ^{Yng}BMCs (42 ± 4.9 hr) and ^{Old}BMCs (58.9 ± 8.9 hr) on days 1–3 after seeding. (**d**) The saturation density of different age-derived BMCs. Saturation density (cells/cm^2) was lowest in ^{Neo}BMCs ($5.5\pm0.07\times10^4$) as compared to ^{Yng}BMCs ($3.5\pm0.1\times10^4$) and ^{Old}BMCs ($2\pm0.1\times10^4$). The values are the means $\pm$ standard error of the mean (SEM) from at least three separate experiments.

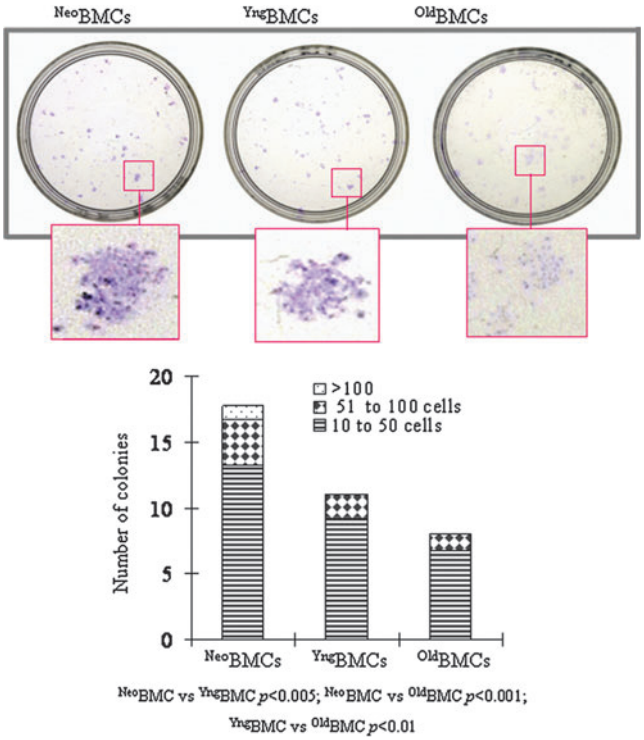

FIG. 2. Effect of age on clonogenicity of bone marrow stromal cells (BMCs). Cells were seeded at a density of 10 cells/cm^2 and cultured with low-glucose Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) for 14 days. Subsequently, the cells were fixed with methanol and stained with Giemsa solution. (**a**) Representative images of number and size of microscopic colonies obtained from expanded ^{Neo}BMCs, ^{Yng}BMCs, and ^{Old}BMCs. (**b**) Quantification of the number of microscopic colonies and cell count within a colony obtained from BMCs of the indicated age. The number of colonies and the number of cells constituting individual colony diminished significantly with aging ($p < 0.01$ vs. ^{Yng}BMCs and $p < 0.001$ vs. ^{Neo}BMCs). The values shown in the graph are the means ± standard error of the mean (SEM) from at least three separate experiments.

proliferation of different age-derived BMCs using specific pharmacological Y5R blocker (Fig. 4a). Nevertheless, pro-proliferative effects of 10 nM NPY were abolished by pretreatment of the cells with 0.25 μM CGP, a specific Y5R blocker in BMCs from all the age groups (Fig. 4a) which clearly showed that interaction of NPY with Y5R was important for BMCs proliferation. Treatment of the cells with CGP71683A alone did not significantly change cell proliferation, except that its effect was more pronounced in ^{Yng}BMCs (Fig. 4b).

Transgenic overexpression of Y5R in ^{Old}BMCs and role of Erk1/2 signaling

To study whether transgenic overexpression of Y5R in ^{Old}BMCs could restore their response to $NPY_{3–36}$ treatment for improved proliferation, the human Y5R transgene was transfected into ^{Old}BMCs by lipofection. Successful transgenic overexpression of Y5R in the transfected cells was confirmed by real-time PCR (Fig. 4c). Y5R transgenic overexpression was significantly increased in Y5R receptor gene-transfected ^{Old}BMCs as compared to GFP gene-transfected ^{Old}BMCs as a control. Using these gene-transfected ^{Old}BMCs, we examined the pro-proliferative effects of $NPY_{3–36}$ treatment which is a known Y2R and Y5R specific agonist. Whereas proliferation of ^{Old}BMCs after Y5R gene transfection in the absence of $NPY_{3–36}$ treatment remained unchanged, treatment of Y5R gene transfected ^{Old}BMCs with 10 nM $NPY_{3–36}$ significantly increased their proliferation as compared to the GFP gene–transfected control ^{Old}BMCs either with or without $NPY_{3–36}$ treatment (Fig. 4d).

Next we examined the mechanism of proliferation of Y5R gene–transfected ^{Old}BMCs in response to $NPY_{3–36}$ treatment (Fig. 5). Given that extracellular signal-regulated kinase 1/2 (Erk1/2) is important in the regulation of proliferative activity in BMCs,[32] we explored the possible involvement of Erk1/2 in mediating the stimulatory effects of $NPY_{3–36}$ on Y5R gene–transfected ^{Old}BMCs, using GFP gene–transfected ^{Old}BMCs as controls. The cells were incubated with CGP (specific Y5R blocker) or 50 μM PD98059 (a specific pharmacological inhibitor of Erk1/2). We observed that 10 nM $NPY_{3–36}$ activated both Erk1 as well as Erk2 in Y5R-transfected ^{Old}BMCs as compared to GFP-transfected ^{Old}BMCs however, with more dramatic changes in Y5R transfected-^{Old}BMCs (Fig. 5a–d).

Discussion

We have demonstrated that the growth characteristics of the BMCs decline with physiological aging with an associated reduction in Y5R expression in ^{Old}BMCs, which curtailed their response to NPY stimulation and significantly reduced Erk1/2 activation. Furthermore, our data avidly supported transgenic overexpression of Y5R as a remedial measure to enhance stimulation of ^{Old}BMCs by $NPY_{3–36}$ to restore their proliferative activity significantly.

The stem/progenitor cells from young donors undergo controlled division for self-renewal and differentiate to produce a large number of healthy mature cells, and therefore differ from stem/progenitor cells from aging donors who possess a limited capacity of self-renewal and differentiation. While characterizing BMCs from various age groups for their culture characteristics with special focus on proliferation potential, we observed that proliferation potential of BMCs declined with physiological aging. Although Dt was no different between ^{Yng}BMCs and ^{Old}BMCs on 1–3 days after cell seeding, saturation density of ^{Old}BMCs was lower than that of both ^{Neo}BMCs and ^{Yng}BMCs. BMCs constitute a heterogeneous population of cells that differ in their surface marker expression and differentiation potential.[33] BMCs also show variable size distribution wherein the ratio of large cells increases with aging.[13] We observed that there were more large cells in the ^{Old}BMCs culture than in the ^{Neo}BMCs and ^{Yng}BMCs cultures. Therefore, contact inhibition in ^{Old}BMCs might have been responsible for their poor proliferative activity more than in ^{Neo}BMCs and ^{Yng}BMCs after cell seeding.

Given that colony formation and the number of cells including a colony are the specific indicators and accurate determinants of proliferative capacity of the cells, we observed that both of these indicators declined considerably in ^{Old}BMCs. Previous studies have demonstrated that colony formation critically depended on the ability of single cells to

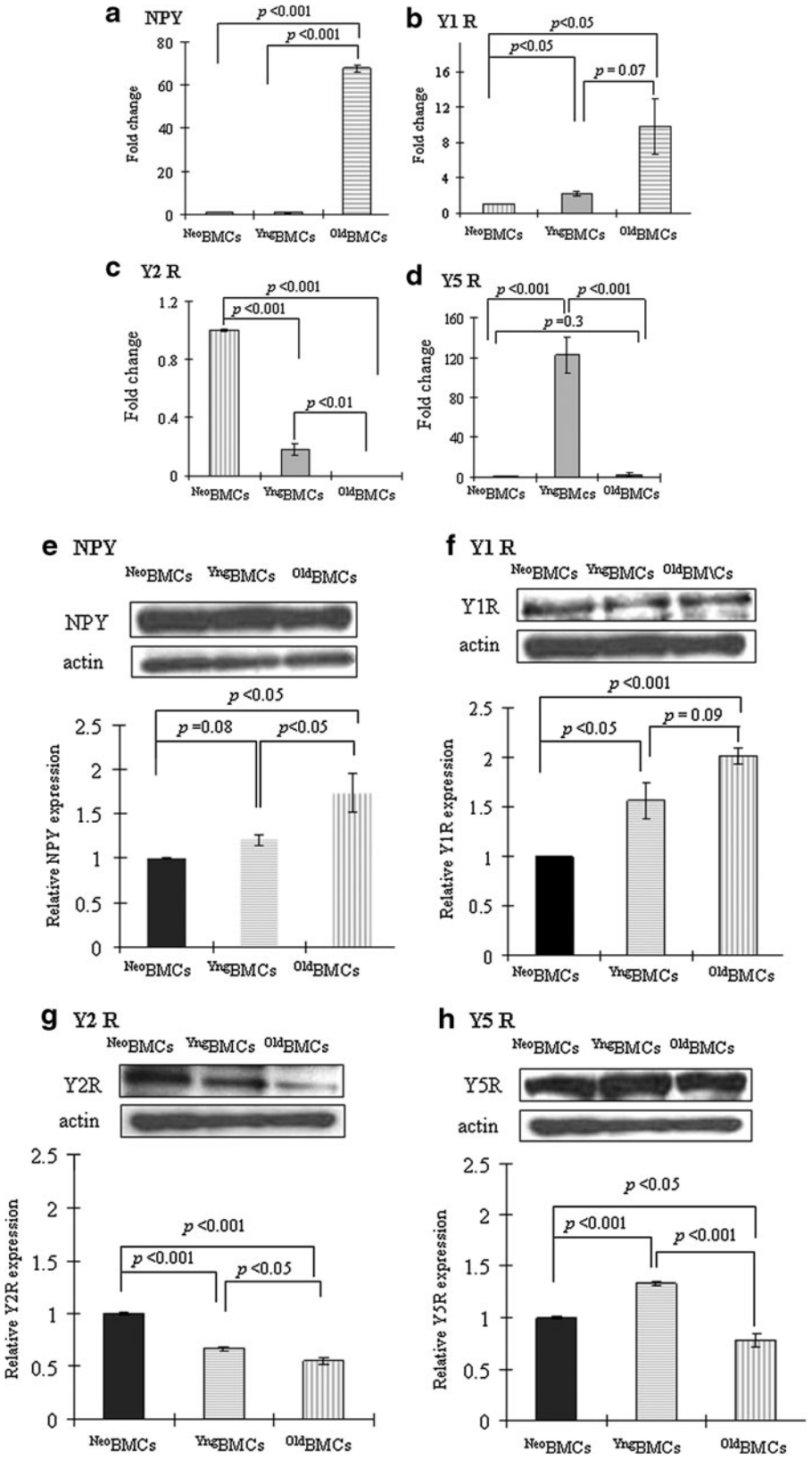

FIG. 3. Expression profile of neuropeptide Y (NPY) and its primary receptors in bone marrow stromal cells (BMCs). (**a–d**) Real time-PCR analysis and (**e–h**) western blot analysis for NPY(**a**, **e**), Y1 receptor (Y1R) (**b**, **f**), Y2R(**c**, **g**), and Y5R (**d**, **h**) in different age-derived BMCs. Values are mean±standard deviation (SD) from at least three separate experiments. Whereas NPY gene expression was 69- and 130-fold higher in ^{old}BMCs as compared to ^{Neo}BMCs and ^{Yng}BMCs, respectively, Y2R and Y5R gene expression showed 28- and 130-fold decline in ^{Old}BMCs. Western blot studies showed a similar trend in the protein expression of NPY and its receptors Y2R and Y5R between ^{Old}BMCs and its younger counterparts. On the contrary, Y1R gene and protein expressions were higher in ^{Old}BMCs.

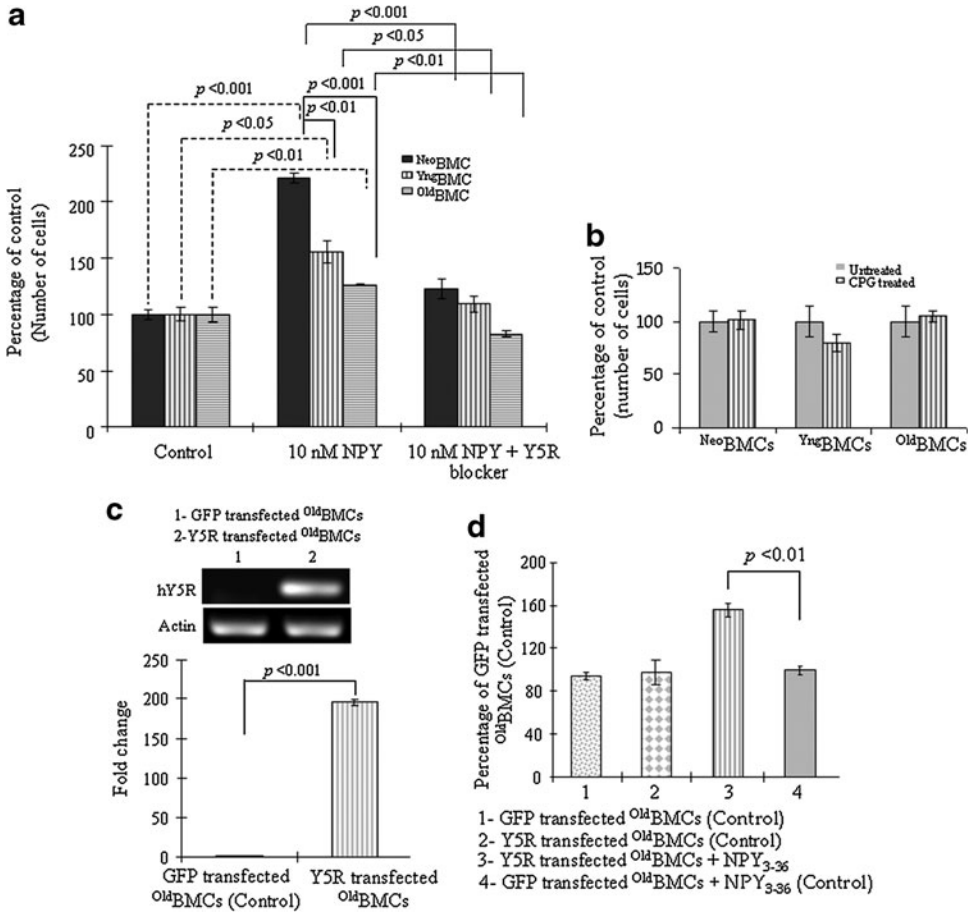

FIG. 4. Neuropeptide $Y_{3–36}$ ($NPY_{3–36}$)/Y5 receptor (Y5R) interaction in ^{Old}BMCs restores their proliferative activity. (**a**) Proliferation potential of bone marrow stromal cells (BMCs) derived from different age groups of donors, i.e., ^{Neo}BMCs, ^{Yng}BMCs, and ^{Old}BMCs, in response to stimulation with 10 nM $NPY_{3–36}$. The cells were seeded at a density of 5×10^3 cells/cm^2 and cultured with low-glucose Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) for 24 hr. After 24 hr, the culture medium was replaced with low-glucose DMEM plus 0.5% FBS. After 24 hr incubation, the cells were cultured with low-glucose DMEM plus 0.5% FBS including 10 nM NPY and Y5R blocker. The cells were counted 10 days after their respective treatment. Significantly higher proliferative activity was observed in ^{Neo}BMCs and ^{Yng}BMCs as compared to ^{Old}BMCs. Pretreatment of the cells with Y5R-specific blocker CPG abolished the stimulatory effect of $NPY_{3–36}$ treatment, which resulted in significant decline in cell proliferation. Nontreated native cells from each age group were used as controls. The values are the means ± standard error of the mean (SEM) from three separate experiments. (**b**) Treatment of the cells with CPG alone insignificantly reduced the proliferative activity of the cells except in each of the age group except that it was more pronounced in ^{Yng}BMCs. Untreated controls in **b** were the cells from respective age group without CPG treatment. (**c**) Real-time PCR analysis for the transgenic overexpression of human Y5R in ^{Old}BMCs using green fluorescent protein (GFP) gene–transfected ^{Old}BMCs as a control. The Y5R gene and GFP gene were transfected in ^{Old}BMCs by lipofection. Significantly higher human Y5R expression was achieved in Y5R gene-transfected ^{Old}BMCs as compared to the controls. (**d**) The proliferation potential of Y5R gene-transfected ^{Old}BMCs after stimulation with 10 nM $NPY_{3–36}$. Y5R gene–transfected cells were seeded at a density of 5×10^3 cells/cm^2 and cultured with low-glucose DMEM supplemented with 10% FBS for 24 hr. After 24 hr, the culture medium was replaced with low-glucose DMEM supplemented with 0.5% FBS. Subsequently, the cells were cultured in low-glucose DMEM supplemented with 0.5% FBS and 10 nM $NPY_{3–36}$. The cells were counted after 4 days. GFP-transfected cells with 10 nM $NPY_{3–36}$ treatment, GFP-transfected cells without $NPY_{3–36}$, treatment and Y5R-transfected cells without $NPY_{3–36}$ treatment were used as controls. Cell proliferation was significantly improved in Y5R-transfected cells without $NPY_{3–36}$ as compared to the controls. The values shown in the graph represent the means±SEM. Data are representative of at least three separate experiments.

divide after attachment in the culture besides the growth speed during incubation period, both of which were compromised during physiological aging.[34] The age-related decline in colony formation in ^{Old}BMCs as compared to parallel cultures of their younger counterparts was apparent very early during their culture. Neonate-derived cell cultures gave rise to the highest percentage of colonies with significant proliferative abilities, whereas cell cultures from aging animals bone marrow gave rise to the lowest percentage of large colonies. These data were supported by previous studies.[34]

Neuropeptides are known to affect multiple cell functions, including cell proliferation.[22] Therefore, we hypothesized that the NPY/NPY receptor system could be exploited to

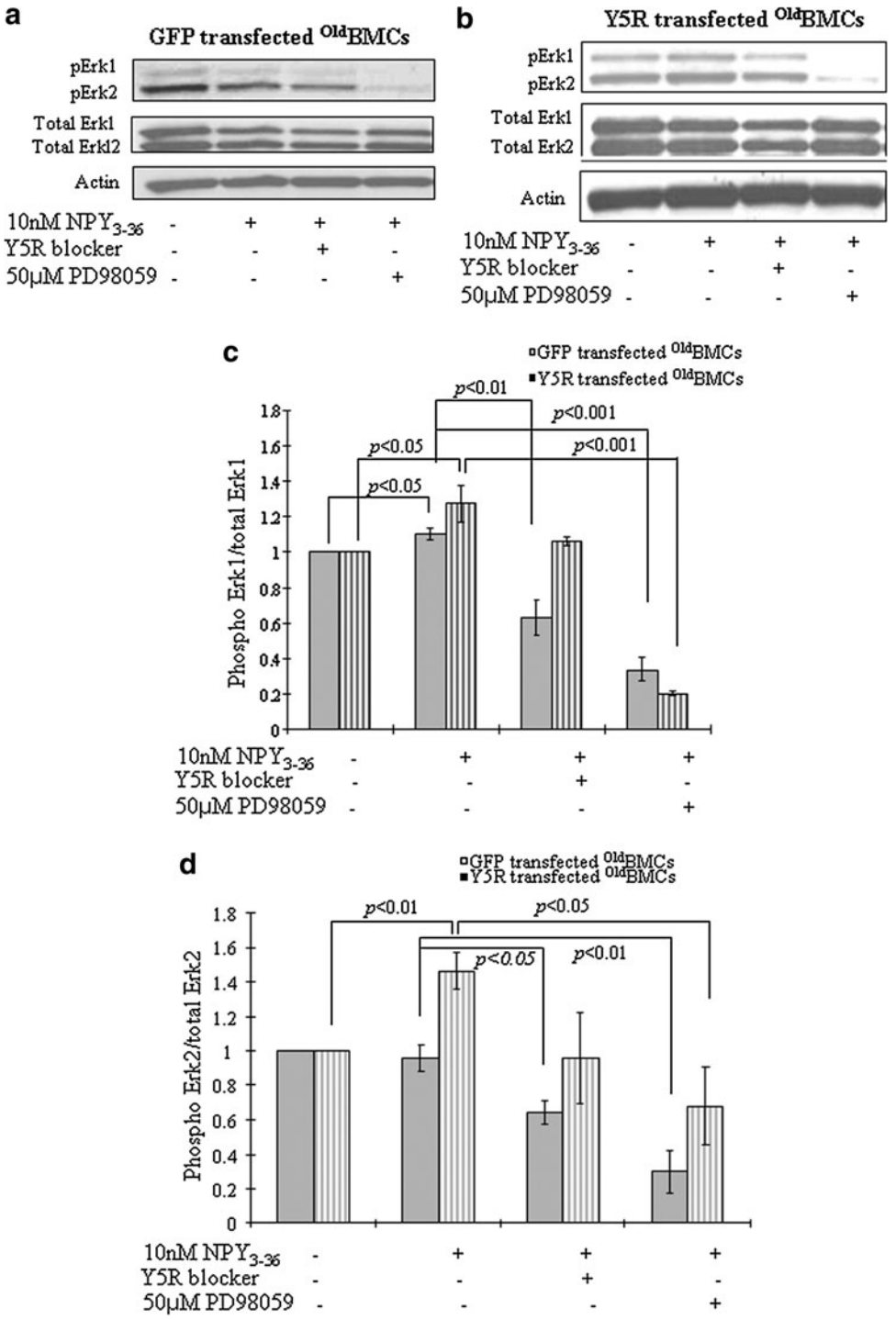

FIG. 5. $NPY_{3–36}$ promotes phosphorylation of extracellular signal–regulated kinases 1/2 (Erk1/2) in neuropeptide Y Y5 receptor (Y5R) gene–transfected ^{Old}BMCs. Representative western blots of green fluorescent protein (GFP) gene–transfected (**a**) (control) and Y5R gene–transfected ^{Old}BMCs (**b**) after their stimulation with $NPY_{3–36}$ showing phosphorylation/activation of Erk1 and Erk2 in replicate samples in comparison with the results of reprobing the same blot for total Erk1/2 expression. (**c–d**) Densitometry of western blots using the AlphaEaseFC from three experiments. Ratios represent the increase over control levels of phosphorylated Erk1 (**c**) and Erk2 (**d**) normalized to the level of total Erk1 and total Erk2, respectively, in GFP gene–transfected (control) and Y5R gene–transfected ^{Old}BMCs. Values were expressed as means ± standard error of the mean (SEM) of three experiments.

restore proliferation potential of ^{Old}BMCs. The published data show NPY/Y1R ligand–receptor interaction influences the capacity of various types of cells to proliferate.[18,20,26] More recently, NPY/Y1R interaction has been implicated as a determinant of the self-renewal capacity of embryonic stem cells that extensively express NPY as well as its primary receptors Y1R and Y5R.[35] Antagonizing either of the receptors resulted in significant loss of self-renewal. Contrary to these observations, we found that despite significantly higher-level expression of NPY and Y1R in ^{Old}BMCs as compared to both ^{Neo}BMCs and ^{Yng}BMCs, ^{Old}BMCs had the longest doubling time (Fig. 1).

These results implied the irrelevance of NPY/Y1R ligand–receptor interaction for induction of BMC proliferation. However, in this regard, it would be prudent that a possible role of dipeptydyl peptidase-IV (DPP-IV) should be determined in different age groups of BMCs. DPP-IV is known to terminate the Y1R activity of NPY by cleavage of its tyrosine 1 to proline 2 and generates an Y2R and Y5R agonist from NPY.[36] Previous studies have already shown that DPP-IV was decreased in the spleens derived from mice.[37] We observed that NPY and Y1R expression increased, whereas Y2R and Y5R decreased in ^{Old}BMCs as compared to ^{Neo}BMCs and ^{Yng}BMCs. Hence, in-depth future studies would be required to determine a regulatory role of DPP-IV in the expression of NPY receptors and the enzymes involved in posttranslational processing of the peptide in BMCs during aging.

Although Y2R expression diminished with age (Figs. 3 and 4), more recent studies have shown that BMCs from Y2R knockout mice and wild-type mice were similar in proliferative capacity, albeit with higher osteogenic and adipogenic differentiation potential in $Y2R^{-/-}$ mice BMCs.[25] In other cell types, such as neuroblastoma cells, however, antagonizing Y2R was pro-apoptotic and also negatively influenced their proliferative activity.[38] In the same context, we have recently shown that specific interaction of NYP with Y5R promoted cell growth and migration through cycle adenosine monophasphate (cAMP) inhibition and Erk1/2 phosphorylation/activation in breast cancer cell lines.[39]

Our laboratory has also reported that NPY/Y5R interaction induced re-entry of cardiomyocytes into the cell cycle and promoted their cytokinesis and mitosis.[40] Extrapolating these data to the BMCs, we focused on NPY/Y5R ligand–receptor interaction as a possible corrective measure to treat aging-associated diminished proliferative activity of ^{Old}BMCs. We observed significantly higher expression of NPY in ^{Old}BMCs, however, with poor autocrine effect on ^{Old}BMCs due to age-associated regression of Y5R expression. The pro-proliferative effect of NPY was mediated through Y5R and was determined by loss- and gain-of-function approaches by blocking Y5R with specific blocker and by transgenic overexpression of Y5R in ^{Old}BMCs.

Our results showed that overexpression of Y5R in ^{Old}BMCs followed by stimulation with $NPY_{3–36}$ significantly increased their proliferation as compared to control cells (GFP-transfected ^{Old}BMCs) with concomitant activation of Erk1/2 in the Y5R-overexpressing ^{Old}BMCs. NPY increases Erk1/2 phosphorylation/activation in a variety of cell lines.[39,41,42] Antagonizing NPY using specific small interfering RNA (siRNA) significantly reduced Erk1/2 activation in neuroblastoma cells with simultaneous reduction in cell proliferation.[38] These results suggested that NPY improved the proliferation potential of ^{Old}BMCs with transgenic Y5R that involved activation of the ERK signaling pathway.

Despite these interesting data, further studies would be required to perform sublineage analysis on BMCs from various age groups to assess the expression of NPY as well as Y1, Y2, and Y5 receptors. However, given the heterogeneous nature of BMCs, and lack of specific markers to identify plastic surface–adherent MSCs, it would require intensive in-depth studies to purify different sublineages and characterize each one of the sublineages for the expression of NPY and its receptors. The present study only focused on heterogeneous BMCs and not its sublineages.

In conclusion, the proliferation potential of BMCs declines with age. NPY treatment subsequent to Y5R overexpression in ^{Old}BMCs rejuvenates their growth characteristics.

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Author Disclosure Statement

The authors have nothing to declare.

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