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Neuropeptide Y enhances proliferation and prevents apoptosis in rat bone marrow stromal cells in association with activation of the Wnt/β-catenin pathway in vitro

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ABSTRACT

Neuropeptide Y (NPY) exhibits a critical but poorly understood regulatory signaling function and has been shown to promote proliferation, vascularization and migration in several types of cells and tissues. However, little is known about the specific role of NPY in the proliferation and apoptosis of bone marrow stromal cells (also known as bone marrow-derived mesenchymal stem cells, BMSCs), which contain a subpopulation of multipotent skeletal stem cells. Based on BrdU incorporation tests, Cell Counting Kit-8, flow cytometry, quantitative polymerase chain reaction and western blotting, we showed that NPY significantly promoted the proliferation of BMSCs in a concentration-dependent manner, with a maximal effect observed at a concentration of 10^{-10} M for pro-proliferative and 10^{-12} M for anti-apoptotic activities. Furthermore, NPY significantly increased the percentage of cells in S and G2/M phases. In addition, NPY exhibited a protective effect after 24 h of serum starvation as illustrated by a reduction in the apoptosis rate, degree of nuclear condensation, and expression of apoptosis markers, including caspase-3, caspase-9 and Bax mRNA expression. NPY also increased the mRNA and protein expression levels of canonical Wnt signaling pathway proteins, including β-catenin and c-myc, during the induced proliferative and anti-apoptotic processes. However, the proliferative and anti-apoptotic activities of NPY were partially blocked by both PD160170 (1 μM) and DKK1 (0.2 μg/mL). These compounds also blocked the mRNA and protein expression of β-catenin, p-GSK-3β and c-myc. Therefore, the results of the present study demonstrated that NPY exerts a proliferative and protective effect on BMSCs in a dose- and time-dependent manner in vitro, and importantly, these effects may be mediated via its Y1 receptor and involved in activation of the canonical Wnt signaling pathway.

1. Introduction

NPY is a highly conserved 36-amino-acid neuropeptide that is broadly expressed in both the central and peripheral nervous systems (Allen et al., 1983; Tatemoto et al., 1982). NPY activates a group of six NPY G protein-coupled receptors (Y1R–Y6R), of which Y1R and Y2R are the most abundantly expressed in mammals (Movafagh et al., 2006; Lee et al., 2010; Teixeira et al., 2009). Previous studies have demonstrated that NPY regulates bone homeostasis through Y receptors expressed in bone marrow cells (Lee et al., 2010; Teixeira et al., 2009; Lee and Herzog, 2009; Liu et al., 2016), and NPY-deficient mice have significantly impaired bone marrow dysfunction, suggesting a protective role of NPY in the bone marrow microenvironment (Park et al., 2015). Researchers have also found that implanting sensory nerve tracts into tissue-engineered bone can significantly improve the early expression of Y1R and osteogenesis, and the same dramatic effect may be manifested by NPY through its stimulation of the proliferation, migration and differentiation of bone marrow stromal cells (BMSCs) (Park et al., 2015; Chen et al., 2010). At the same time, our previous studies reported that NPY stimulates osteoblastic differentiation and migration of BMSCs (Liu et al., 2016), and we observed that another neuropeptide, substance P, could regulate BMSC osteogenic differentiation, proliferation and apoptosis, thus promoting fracture healing (Fu et al., 2014, 2015). However, the proliferative effect of NPY on BMSCs is still controversial. NPY was reported to inhibit the proliferation of BMSCs, and BMSCs isolated from Y1 −/− mice had a significantly greater proliferative ability than cultures isolated from wild-type mice (Lee et al., 2010). In contrast, other reports have suggested that NPY may stimulate the proliferation of BMSCs in vitro (Igura et al., 2011). Therefore, the specific role of

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Table 1
Primers for qPCR.

Target gene	Primer sequence	Length (bp)
cyclin D1	F: 5′- TGCGTGCAGAGGGAGATTG-3′ R: 5′-AGCGGGAAGACCTCCTCTTC-3′	101 bp
p53	F: 5′- GAGCTGAATGAGGCCTTGGA-3′ R: 5′-GACTGGCCCTTCTTGGTCTTC-3	101 bp
caspase 3	F: 5′-AGTGGAGGCCGACTTCCTGTA-3′ R: 5′-TGGCGCAAAGTGACTGGAT-3′	101 bp
caspase 8	F: 5′-GTTTTGGATGAGGTGACCATCA-3′ R: 5′-TACCCCCGAGGTTTGCTCTT-3′	116 bp
Bax	F: 5′-CCCACCAGCTCTGAACAGTTC-3′ R: 5′-TCTCCCCAGCCATCCTCTCT-3′	88 bp
c-myc	F: 5′-AGTGGAGGCCGACTTCCTGTA-3′ R: 5′-TGGCGCAAAGTGACTGGAT-3′	101 bp
β-catenin	F: 5′- TGACGGTCAGTACGCCATGA-3′ R: 5′-GGCATCAAACTGCGTGGAT-3′	102 bp
GAPDH	F: 5′-AGGGCTGCCTTCTCTTGTGA-3′ R: 5′-AACTTGCCGTGGGTAGAGTCA-3′	110 bp

NPY in proliferation and apoptosis of BMSCs, which are critical for maintaining an appropriate number of BMSCs in the body and play an important role in osteogenesis, has not been fully characterized and needs further study.

A fast-growing field has linked the Wnt pathway with bone development, remodeling, and repair (Rawadi and Roman-Roman, 2005; Monroe et al., 2012; Westendorf et al., 2004). The best known Wnt signaling pathway involves β-catenin (commonly called the canonical pathway). Wnts are secreted glycoproteins that bind to receptor complexes such as low-density lipoprotein receptor-related protein (LRP) 5/6, which are disrupted and inhibit the activity of glycogen synthase kinase-3β (GSK-3β), these changes promote β-catenin translocation into the nucleus where it can regulate gene expression. Dickkopf-related protein-1 (DKK1) inhibits Wnt signaling by binding to LRP 5/6 and is considered a specific inhibitor of the canonical Wnt signaling pathway (Bafico et al., 2001). It has been shown that canonical Wnt signaling is a key player in the proliferation and survival of osteoblasts (Westendorf et al., 2004) and the proliferation and osteogenic differentiation of BMSCs, whereas DKK1 negatively regulates those effects (Gregory et al., 2005; Boland et al., 2004). However, the extent of canonical Wnt signaling participation in BMSC proliferation and apoptosis is still poorly understood.

Based on the roles of NPY and the Wnt/β-catenin pathway in regulating osteogenesis, we hypothesized that NPY would enhance proliferation and decrease apoptosis in serum-deprived BMSCs in vitro and that this activity may be related to the Wnt/β-catenin pathway. First, by using BrdU incorporation tests, Cell Counting Kit-8 (CCK-8) assays and flow cytometry, we analyzed the optimal effective concentration of NPY during proliferation and apoptosis, and then, to determine whether the Wnt/β-catenin pathway is responsible for those activities, we examined the genes or proteins associated with this pathway using the

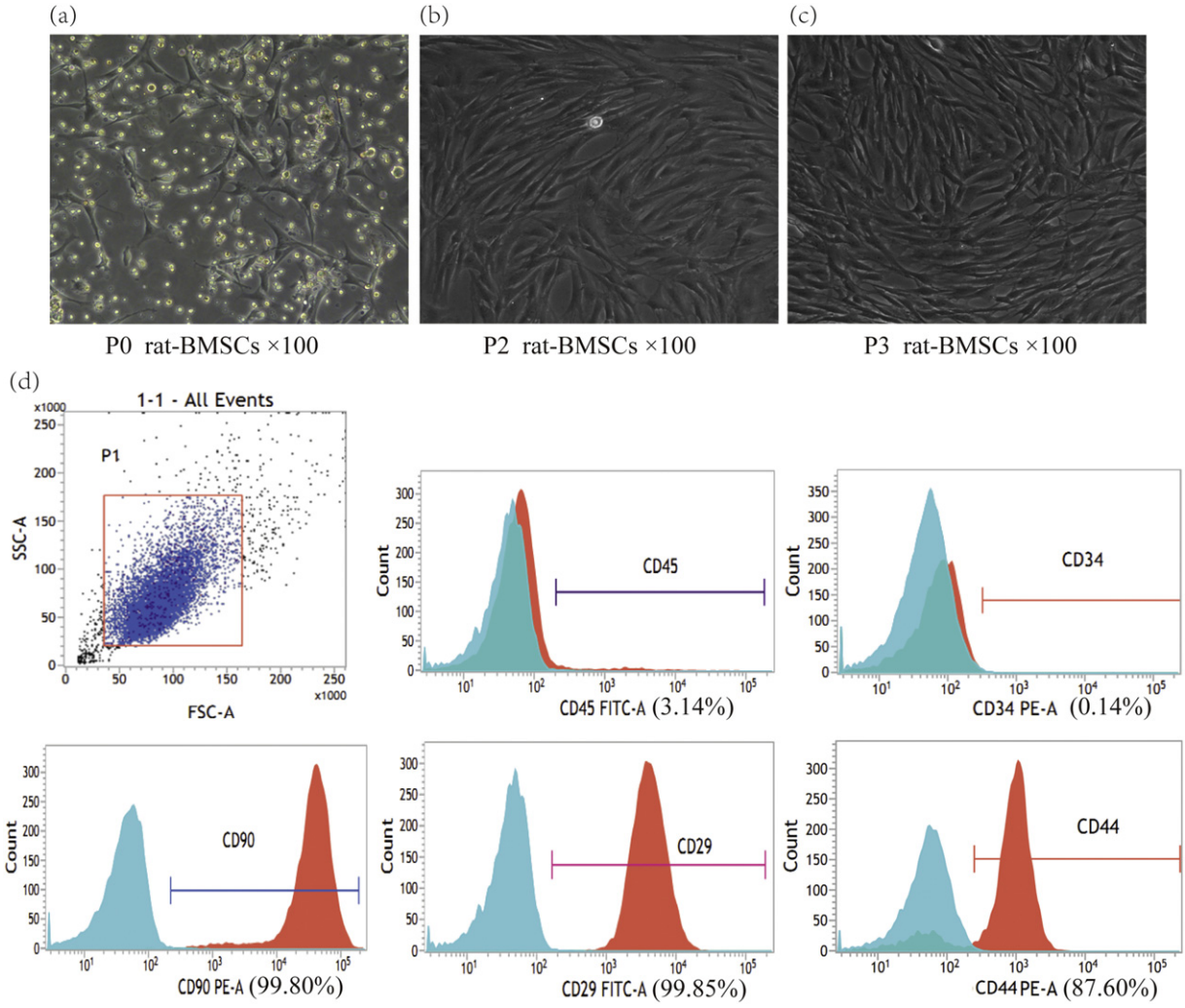

Fig. 1. Morphology and identification of BMSCs. Primary BMSC cultures at (a) P0 (the initial culture), (b) P2 (the second passage), and (c) P3 (the third passage) (magnification, 100×). Identification of the surface marker expression patterns (CD90, CD29, CD44, CD34, CD45) of BMSCs using flow cytometry (d).