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The P75 neurotrophin receptor regulates proliferation of the human MG63 osteoblast cell line

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ABSTRACT

The 75 kDa transmembrane protein, $p75^{NTR}$, is a marker of mesenchymal stem cells (MSCs). Isolated MSCs are capable of differentiating into osteoblasts, but the molecular function of $p75^{NTR}$ in MSCs and osteoblasts is poorly understood. The aim of this study was to examine the function of $p75^{NTR}$ in the human MG63 osteoblast cell line compared to the murine MC3T3E-1 pre-osteoblast cell line.

MG63 cells and MC3T3-E1 cells expressing exogenous $p75^{NTR}$ protein (denoted as p75-MG63 and p75GFP-E1, respectively) were generated to compare osteogenic differentiation and cell proliferation abilities. Overexpression of $p75^{NTR}$ induced alkaline phosphatase activity and the mRNA expression of osteoblast-related genes such as osterix and bone sialoprotein in both p75-MG63 and p75GFP-E1. Interestingly, exogenous $p75^{NTR}$ stimulated cell proliferation and cell cycle progression in p75GFP-E1, but not in p75-MG63.

To elucidate any different effects of $p75^{NTR}$ expression on osteogenic differentiation and cell proliferation, we examined the mRNA expression of tropomyosin receptor kinase (trk) genes (trkA, trkB, trkC) and Nogo receptor (NgR), which are binding partners of $p75^{NTR}$. Although trkA, trkB, and trkC were detected in both p75-MG63 and p75GFP-E1, only NgR was detected in p75-MG63. We then used the K252a inhibitor of the trks to identify the signaling pathway for osteogenic differentiation and cell proliferation. Inhibition of trks by K252a suppressed $p75^{NTR}$-mediated osteogenic differentiation of p75GFP-E1, whereas deletion of the GDI domain in $P75^{NTR}$ from the p75-MG63 produced enhanced cell proliferation compared to p75-MG63. These results suggest that $p75^{NTR}$ signaling associated with trk receptors promotes both cell proliferation and osteoblast differentiation, but that $p75^{NTR}$-mediated proliferation may be suppressed by signaling from the $p75^{NTR}$/NgR complex.

1. Introduction

Continuous remodeling in bone involves a balance between bone-forming osteoblasts derived from mesenchymal stem cells (MSCs) and bone-resorbing osteoclasts derived from hematopoietic stem cells (HSCs). MSCs have been isolated from bone marrow, adipose tissue, umbilical cord, and dental-related tissues such as dental follicle, periodontal ligament, and dental pulp from adult and deciduous teeth (Gronthos et al., 2000; Honda et al., 2007, 2010, 2011; Pittenger et al., 1999; Seo et al., 2004). As a result, the strategy of engineering bone growth by using MSCs transplantation has become widely accepted (Ishikane et al., 2013). However, the proper utilization of MSCs/progenitors for clinical applications requires an integrated understanding of the molecular mechanisms involved in the differentiation from MSCs to osteoblasts.

Cell surface markers have been used for the selection of MSCs, but a single cell surface marker that exclusively defines MSCs has not been discovered (Barry and Murphy, 2004). Recently, the 75 kDa transmembrane protein, $p75^{NTR}$, also referred to as nerve growth factor receptor (NGFR), tumor necrosis factor receptor superfamily member 16 (TNFRSF16), and CD271, has been described as a marker

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of MSCs in bone marrow, adipose tissue, and umbilical cord (Christodoulou et al., 2013; Dmitrieva et al., 2012). We also identified $p75^{NTR+}$ cells isolated from deciduous dental pulp (DDP-$p75^{NTR+}$ cells) that showed different characteristics from bone marrow-derived MSCs, which could differentiate into adipogenic and osteogenic lineages (Mikami et al., 2011; Quirici et al., 2002). However, although the differentiation of DDP-$p75^{NTR+}$ cells into osteoblasts and adipocytes was largely inhibited, a fraction of cells showed some such abilities (Mikami et al., 2011). Interestingly, the presence of cells with the DDP-$p75^{NTR+}$ phenotype diminishes gradually with culture (Mikami et al., 2011), a phenomenon also observed by others (Margossian et al., 2012), and cells that had lost $p75^{NTR}$ expression were then capable of differentiating into osteoblasts and adipocytes (Mikami et al., 2011). Furthermore, although DDP-$p75^{NTR+}$ cells showed high rates of proliferation in our hands, a slowly proliferating subpopulation of MSCs that highly expressed $p75^{NTR}$ were identified from umbilical cord blood (Ju et al., 2013). In contrast, we did not find $p75^{NTR+}$ cells in human periodontal ligament-derived cells (Honda et al., 2013) and the use of $p75^{NTR}$ failed to isolate MSCs from umbilical cord blood (Watson et al., 2013). Taken together, these observations indicated that the properties of $p75^{NTR+}$ cells vary among different tissues or cells, which does not aid our understanding of the function of $p75^{NTR}$ in MSCs and osteoblasts.

A relationship between signaling from $p75^{NTR}$ and tropomyosin receptor kinase (trk) has been documented in the nervous system (Kaplan and Miller, 2000; Patapoutian and Reichardt, 2001), but the function of $p75^{NTR}$ in MSCs and osteoblasts remains to be clarified. In this study, osteoblast differentiation of the murine C3H10T1/2 MSC cell line was inhibited by exogenous $p75^{NTR}$ expression. Interestingly, expression of the trk receptors (trk A, B, C) was also not detected in the C3H10T1/2 cells, which suggests that trk signaling does not contribute to the $p75^{NTR}$-mediated inhibition of osteogenic differentiation (Mikami et al., 2011). On the other hand, MC3T3-E1 pre-osteoblast cells constitutively expressing exogenous $p75^{NTR}$ show strongly enhanced cell proliferation and osteogenic differentiation (Mikami et al., 2012). MC3T3-E1 cells express all of the trk receptors and the osteogenic differentiation of MC3T3-E1 cells expressing exogenous p75 was inhibited by treatment with the K252a inhibitor of trk tyrosine kinase (Mikami et al., 2012). These observations thus confirm that $p75^{NTR}$ signaling is associated with the trk tyrosine kinase receptor in MC3T3-E1 cells.

Osteoblast-like cells derived from the human MG63 osteoblast cell line have been extensively studied in bone biology research. Comparisons of MC3T3-E1 cells with MG63 cells have revealed some phenotypic differences (Czekanska et al., 2013; Miron et al., 2013); in particular, the cellular proliferation activities and osteogenic differentiation potential (Czekanska et al., 2013). Interestingly, the Nogo receptor, NgR, is expressed in MG63 cells, but not in MC3T3-E1 cells. The aim of this study was therefore to further examine the $p75^{NTR}$-modulated mechanisms of cell proliferation and osteoblast differentiation in MG63 cells compared to those operating in MC3T3-E1 cells containing exogenous $p75^{NTR}$.

2. Materials and methods

2.1. Overexpression of $p75^{NTR}$

MC3T3-E1 and MG63 cells were obtained from the RIKEN cell bank (Ibaragi, Japan). The $p75^{NTR}$ expression plasmids were constructed by standard methods. The human $p75^{NTR}$ and deleted-$p75^{NTR}$ cDNAs (Fig. 5) were prepared by PCR and cloned into pIREShyg (Clontech/Takara Bio, Tokyo, Japan). MG63 cells were plated at a density of 5×10^5 cells/well in a 6-well cell culture dish and were cultured in α-MEM medium (Gibco BRL, Grand Island, NY, USA) with 10% fetal bovine serum (FBS, Japan Bioserum Co., Ltd. Tokyo, Japan) and 1% penicillin–streptomycin (Gibco) for 24 h at 37 °C in the presence of 5% CO_2. Subsequently, the cells were incubated in 2 mL of α-MEM containing 6.25 μL of lipofectamine LTX (Invitrogen, Carlsbad, CA, USA) and 2 μg of plasmid. The empty pIREShyg vector DNA was used as a transfection control. Cells with stable DNA integration were selected by culturing with 200 mg/mL hygromycin (Sigma, St. Louis, MO, USA). Cells expressing $p75^{NTR}$ (p75-MG63) or deleted-$p75^{NTR}$ (p75Del-MG63) were selected from the transfected MG63 cells using the BD FACS Aria (BD Biosciences, San Jose, CA, USA) and a PE-conjugated human $p75^{NTR}$ antibody (BD Biosciences). MG63 cells stably transfected with the empty expression vector cells (Mock-MG63) were similarly prepared and used as transfection control cells.

The stably transfected MC3T3-E1 cells expressing $p75^{NTR}$ fused to GFP (p75GFP-E1) and MC3T3-E1 cells expressing a non-fused GFP (GFP-E1) were established previously (Mikami et al., 2012).

2.2. Cell culture and osteogenic differentiation protocol

Cells stably transfected with each plasmid were grown to confluence in 12-well culture dishes in the growth medium at 37 °C in the presence of 5% CO_2. Subsequently, the cells were subcultured for various periods in osteogenic induction medium containing α-MEM supplemented with 10% FBS, 50 μg/mL L-ascorbate phosphate (Sigma), 10 mM β-glycerophosphate (Sigma), and 100 ng/mL hrBMP-2 (R&D System, Minneapolis, MN, USA). Cells were maintained with fresh osteogenic induction medium every 2 d for the indicated period.

2.3. Observation by fluorescent microscopy

Cells were trypsinized and collected onto slides with a cytocentrifuge and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature. The fixed cells were permeabilized in 0.5% NP-40 in PBS for 15 min at room temperature, and rinsed three times in PBS containing 0.5% bovine serum albumin. The p75-MG63 and Mock-MG63 cells were then incubated for 1 h at 37 °C with PE-conjugated anti-human $p75^{NTR}$ antibody (BD Biosciences). The cells were then washed with PBS and the nuclei were counterstained with 0.2 μg/mL DAPI in Vectashield Antifade (Vector Laboratories). All immunofluorescence images were collected by fluorescence microscopy (Biozero BZ-8000, Keyence, Tokyo, Japan) and the images were analyzed with the Keyence software, BZ analyzer.

2.4. Cell counting assay

Cells were seeded onto 100-mm culture dishes (1×10^4 cells/dish) and cultured for the indicated periods in growth medium. The cells were then trypsinized and resuspended in growth media, whereupon the number of cells was counted using a hemocytometer.

2.5. Cell cycle analysis

Cell cycle analysis was performed using Click-iT™ EdU Flow Cytometry Assay Kits (Invitrogen/Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. In brief, cultured cells were treated with 10 μM EdU (5-ethynyl-2′-deoxyuridine) for 1 h. EdU is a nucleoside analog of thymidine and is incorporated into DNA during active DNA synthesis. The EdU-treated cells were fixed with paraformaldehyde for 15 min, washed once with BSA/PBS buffer, and then permeabilized with a saponin-based buffer for 30 min. The cells were then washed once, treated with the click-reaction mixture containing Pacific