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Upregulated Genes in Contact Co-culture of Osteoblasts and Sympathetic Neuronal Cells

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Abstract: Many works have been done on the interaction between neurons and osteoblasts. However, the cells used in our previous study were derived from mouse and rat, and it is impossible to exclude the possibility of cross-reactivity of the mouse and rat probes Osteoblasts cell line labeled with red fluorescent protein and sympathetic neuronal cell line labeled with green fluorescent protein were generated. A DNA microarray analysis was conducted by using these cell line in co-culture system. It was filtered for genes with a ratio more than 2.0-fold in the biological process category by the search term "axon" and cellular component by the search term "membrane". Eight genes, i.e., neuropilin 1, growth associated protein 43, dopamine receptor D2, plexin A2, disabled 1, ephrin receptor A7, ephrin B2, and fibronectin leucine rich transmembrane protein 3 were found. The molecules expressed on the plasma membrane of osteoblasts were upregulated in contact co-culture with sympathetic neuronal cells. It was filtered for genes with a ratio more than 2.0-fold in the biological process category by the search term "osteoblast" and cellular component by the search term "membrane". Four genes, i.e., gap junction protein, alpha 1, collagen, type VI, alpha 1 and bone morphogenetic protein 4 were found. The molecules expressed on the plasma membrane of neurons were upregulated in co-culture with osteoblasts. These molecules may be candidates for factors that promote osteoblast differentiation and neural differentiation.

Keywords: Osteoblast, Sympathetic Neuronal Cell, Contact Co-culture, Differentiation, Microarray Analysis

1. Introduction

Bone formation by osteoblasts and bone absorption by osteoclasts regulate bone metabolism [1]. Accumulating evidences reveal that the central nervous system controls bone through several osteoblast receptors [2]. For instance, the efferent sympathetic pathway regulates osteoblasts via noradrenaline, which binds to the beta2-adrenergic receptor, and neuropeptide Y, which binds to the Y1 receptor [3]. Fukuda et al. have previously shown that semaphorin 3A from the sensory nerves also regulate osteoblasts [4]. It has been reported that mechanical stimulation increases intracellular Ca2+ concentrations in osteoblasts via P2X receptors by contact co-culture with neurons [5]. In addition, contact culture with dorsal root ganglion neurons induced an increase in Ca2+ levels in osteoblasts [6]. It has been shown that the sympathetic nervous system actively regulates bone resorption and that sympathetic signaling promotes osteoblast proliferation via adrenergic receptors [7, 8]. On the other hand, fenoterol, an agonist of β-adrenergic receptor and adrenaline, does not promote osteoblast differentiation, whereas BMP-2 and adrenaline have been shown to promote osteoblast differentiation better than BMP-2 alone [9].

The nerve axons are present in the vicinity of osteoblasts [10] and sympathetic nerve fibers innervate the periosteum [11]. Several researchers reported that the interaction between osteoblasts and neuronal cells [12-14]. Okubo et al. previously performed a DNA microarray analysis to investigate the relationship between osteoblasts and sympathetic neuronal cells in contact co-culture. Okubo et al. found that the sympathetic neuronal cells promoted osteoblast differentiation and the osteoblasts promoted neuron differentiation [15]. However, the cells used in our previous study were derived from mouse and rat, and it is hard to exclude the possibility of cross-reactivity of the

mouse and rat probes. Osteoblasts cell line labeled with red fluorescent protein (DsRed) and sympathetic neuronal cell line labeled with green fluorescent protein (GFP) were generated. The generated cell lines were used in co-culture system and a DNA microarray analysis was performed.

2. Materials and Methods

2.1. Cell Cultures

The mouse pre-osteoblastic cell line MC3T3-E1 was maintained in α-modified minimum essential medium (αMEM; Wako, Osaka, Japan) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS). The sympathetic neuronal cell line PC12, derived from rat pheochromocytoma, was maintained in high glucose Dulbecco´s modified eagle medium (DMEM; Wako) supplemented with 10% horse serum, 10% FBS, and 1% penicillin/streptomycin. The medium was changed every 3 days. All cultures were maintained at 37°C in humidified air containing 5% CO_2. For the contact co-culture of MC3T3-E1 cells and PC12 cells, DMEM was used [15].

2.2. Retrovirus Preparation and Infection

To isolate the retrovirus, pMX-IRES-GFP or pMX-DsRed were transiently transfected into Plat-E cells (platinum-E retrovirus packaging cell line; Cell Biolabs, Inc.) with ScreenFect A (FUJIFILM Wako Chemicals, Ltd. Osaka, Japan) according to the manufacturer´s protocol. Viral supernatant was collected from the culture media 48 h after transfection. PC12 cells were incubated with viral supernatant (GFP transfected into Plat-E) in the presence of polybrene (10 μg/ml) for 8 h. MC3T3-E1 cells were incubated with viral supernatant (DsRed transfected into Plat-E) in the presence of polybrene (10 μg/ml) for 8 h. The infection efficiency of the retrovirus was determined by GFP or DsRed expression and was always greater than 90% (Figure 1 and Figure 2). We designated PC12 cells transfected with GFP as PC/GF and MC3T3-E1 cells transfected with DsRed as MC/Ds.

2.3. Flow Cytometry

A fluorescence-activated cell sorter (SH800S, Sony Biotechnology) equipped with 488-nm and 561-nm laser was used to detect green and red fluorescence, respectively. 525/50-nm and 600/60-nm emission filters were used to filter green and red fluorescence, respectively. MC/Ds cells were incubated with PC12/GFP. After the incubation for 72 h, these cells were sorted by their respective fluorescent proteins. The post-sorting MC3T3-E1/DsRed (designated as psMC/Ds cells) and the post-sorting PC12/GFP (designated as psPC/GF cells) were obtained.

2.4. DNA Microarray Analysis

The microarray analysis was performed using total RNA samples from MC/Ds, PC/GF, psMC/Ds, and psPC/GF that were incubated for 72 h. The total RNA was extracted from cells using the RNeasy mini kit (Qiagen, Düsseldorf, Germany), and DNA microarray analyses were performed using the 3D-Gene Mouse Oligo chip 24k and Rat Oligo chip 20k (Toray Industries Inc., Tokyo, Japan) according to the manufacturers' instructions. For efficient hybridization, these microarrays have a columnar structure to stabilize the spot morphology and enable microbead agitation. The total RNA was labeled with Cy5 using the Amino Allyl MessageAMP II aRNA Amplification Kit (Thermo Fisher Scientific). The Cy5-labeled aRNA pools were mixed with hybridization buffer, and hybridized for 16 h according to the manufacturer's protocols (www.3d-gene.com). The hybridization signals were obtained using a 3D-Gene Scanner (Toray Industries Inc.), and were processed by the 3D-Gene Extraction software (Toray Industries Inc.). Detected signals for each gene were normalized by a global normalization method (the median of the detected signal intensity was adjusted to 25). Gene ontology (GO) analysis using GeneCodis was performed for the significant genes. Each reaction was performed in triplicate on three individual samples.
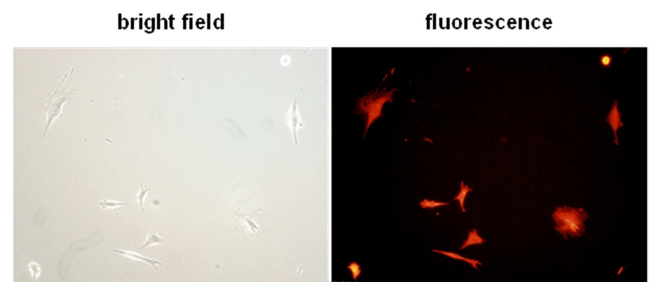

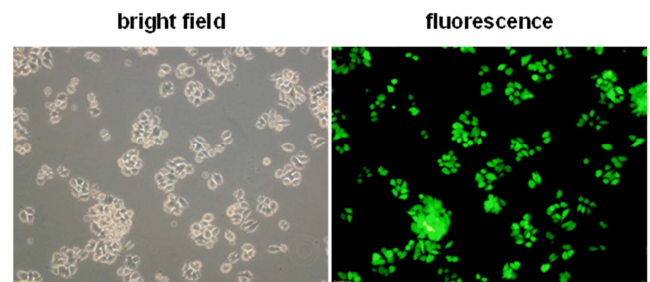**Figure 1.** *MC3T3-E1 cells express DsRed protein.*

Figure 2. *PC-12 cells express GFP protein.*

3. Results and Discussion

We performed DNA microarray analysis of each sample including MC/Ds incubated with PC12 for 0h and psMC/Ds incubated with PC12 for 72h to compare the gene expression patterns of osteoblasts before and after co-culture with sympathetic neuronal cells using the Mouse Oligo chip were performed. In humans, peripheral nerves are found in bone tissue, not only in the periosteum but also in the bone marrow [15]. Okubo et al. have previously shown that contact between neurons and osteoblasts promotes reciprocal differentiation, suggesting that the contact between neurons and osteoblasts is important [14]. Therefore, the hypothesis that surface

molecules on osteoblasts stimulate the differentiation in neuronal cells is proposed. It was filtered for genes with a ratio more than 2.0-fold in the biological process category by the search term "axon" and cellular component by the search term "membrane". Eight genes, i.e., neuropilin 1 (Nrp1), growth associated protein 43 (Gap43), dopamine receptor D2 (Drd2),

plexin A2 (Plxna2), disabled 1 (Dab1), ephrin receptor A7 (Epha7), ephrin B2 (Efnb2), and fibronectin leucine rich transmembrane protein 3 (Flrt3) were found. However, since receptors should be excluded as molecules that promote neuronal differentiation, molecules excluding receptors are listed in Table 1.

Table 1. *Upregulated neuronal axon and plasma membrane-related genes expressed by murine osteoblasts using Mouse Oligo chip.*

Symbol	Description	Ratio (72 h/ 0 h)*
Gap43	growth associated protein 43	6.86
Dab1	disabled 1	2.91
Efnb2	ephrin B2	2.10
Flrt3	fibronectin leucine rich transmembrane protein 3	2.01

*more than 2 times

Gap43 attached to the membrane is a neuron-specific phosphoprotein, which plays critical role in axon growth and synapses functions during neurogenesis [16]. Our finding that the Gap43 gene is expressed in osteoblasts is very interesting because it has never been reported before. Gap43 is expressed on the plasma membrane of osteoblasts and is suggested to be one of the factors that promote neuronal differentiation. Dab1 is essential for neuronal migration and maturation in response to the extracellular protein Reelin [17]. Efnb2 is a crucial regulator of cellular interactions in a multitude of developmental and physiological processes [18] and Efnb2/EphB4 forward signaling induces neuronal differentiation [19, 20]. Flrt3 is a modulator of neurite outgrowth, axon pathfinding, and cell adhesion [21]. Taken together, Gap43, Dab1, Efnb2, and Flrt3, molecules expressed on the plasma membrane of osteoblasts, which were upregulated in contact co-culture with sympathetic neuronal cells, and may be candidates for factors that promote neural differentiation.

DNA microarray analysis of each sample including PC/GF and psPC/GF to compare the gene expression patterns of sympathetic neuronal cells before and after co-culture with osteoblasts using the Rat Oligo chip were also performed. The hypothesis that surface molecules on osteoblasts stimulate the differentiation in neuronal cells is proposed. It was filtered for genes with a ratio more than 2.0-fold in the biological process category by the search term "osteoblast" and cellular component by the search term "membrane". 4 Four genes, i.e., gap junction protein, alpha 1 (Gja1), collagen, type VI, alpha 1 (Col6a1), and bone morphogenetic protein 4 (Bmp4) were found. However, since receptors should be excluded as molecules that promote osteoblast differentiation, molecules excluding receptors are listed in Table 2. Col6A1 is also excluded from the list because it is unlikely to be a molecule that promotes osteoblast differentiation.

Table 2. *Upregulated osteoblast and plasma membrane-related genes expressed by rat sympathetic neuronal cells using Rat Oligo chip.*

Symbol	Description	Ratio (72 h/ 0 h)*
Gja1	gap junction protein, alpha 1	8.90
Bmp4	bone morphogenetic protein 4	2.11

*more than 2 times

Gja1, also known as connexin 43, has an important role in

skeletal homeostasis [22]. Bmp4 is a potent inducer of osteoblast differentiation via RUNX2 regulation [23]. Collectively, Gja1 and Bmp4, molecules expressed on the plasma membrane of neurons, were upregulated in co-culture with osteoblasts. These molecules may be candidates for factors that promote osteoblast differentiation. Two limitations of this study include: first, the cells are derived from rodents; second, the experiments in this study are in vitro experimental systems, so their role in vivo is unknown.

To elucidate the role for neuronal differentiation, it was needed to examine whether the disruption of the molecules (i.e., Gap43, Dab1, Efnb2, and Flrt3) in osteoblasts does not induce neuronal differentiation in contact co-culture with sympathetic neuronal cells. Moreover, to elucidate the role for osteoblast differentiation, it was also needed to examine whether the disruption of the molecules (i.e., Gja1 and Bmp4) in sympathetic neuronal cells does not induce osteoblast differentiation in contact co-culture with osteoblasts.

4. Conclusion

In conclusion, neurons and osteoblasts may promote mutual differentiation under contact culture. It was suggested that molecules present in the plasma membrane of neurons may promote osteoblast differentiation through direct contact with osteoblasts. It was also suggested that molecules present in the plasma membrane of osteoblasts may be in direct contact with neurons and promote neuronal differentiation.

Acknowledgements

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