Original

Gene Expression of Semaphorin 7A During Osteogenic Differentiation in Human Dental Follicle Cells

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(Accepted for publication, April 22, 2016)

Abstract: Semaphorin (Sema) 7A, also known as CDw108, is the only glycophosphatidylinositol (GPI)-linked member of the semaphorin family. It was recently suggested that Sema 7A regulates osteoblast and osteoclast differentiation, and is important for bone homeostasis. The dental follicle is an ectomesenchymal tissue that surrounds developing tooth germ, which contains osteoblastic/cementoblastic lineage committed stem/progenitor cells. The purpose of this study was to examine the gene expression of Sema 7A and its receptors, plexin C1 and β-integrin, in human dental follicle cells (hDFCs) during osteogenic differentiation. We analyzed gene expression profiles between hDFCs cultures with GM and OIM on day 17. Sema 7A was the most up-regulated among the semaphorin family members in OIM culture when compared to GM culture. We confirmed the gene expression of Sema 7A in three hDFCs samples isolated from three donors using real-time PCR. Sema 7A was up-regulated in OIM culture in three hDFC samples. In addition, Sema 7A mRNA showed high levels on days 0 and 17 in hDFCs cultured in OIM, as compared to days 3 and 10, exhibiting bimodal expression during osteoblast differentiation. The expression of plexin C1 and β-integrin was also up-regulated in OIM culture when compared to GM culture. These findings suggest that Sema 7A and its receptors are associated with the osteogenic differentiation of hDFCs.

Key words: Human dental follicle cells, Semaphorin 7A, Osteogenic differentiation, Stem cells.

Introduction

The semaphorins are a large family of phylogenetically similar secreted and membrane-bound proteins, and are divided into eight classes based on sequence similarities and distinct structural features1,2). Semaphorins influence cellular processes such as cell proliferation, differentiation and survival. As a consequence, they are involved in a variety of biological processes, such as axonal guidance3), cardiogenesis4), angiogenesis5), oncogenesis6), immune cell regulation7) and bone homeostasis8).

Semaphorin (Sema) 7A, also known as CDw108, was identified as a 78-80-kDa cell surface protein on red blood cells9,10), and was subsequently characterized as a member of the semaphorin family11,12). Sema7A is the only glycophosphatidylinositol (GPI) -linked member of the semaphorin family13), and its effects are believed to be mediated via two receptors, Plexin C113) and β-integrin subunit14). Sema 7A is broadly expressed in lymphoid cells15), the nervous system16), endothelial cells of blood vessels17), and bone cells18). Recent studies have shown that Sema 7A and its receptors regulate osteoblast and osteoclast differentiation, both important for bone homeostasis19,20).

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Materials and Methods

Cell cultures

hDFCs were obtained from patients aged 18 years after...
obtaining informed consent, as reported previously. Briefly, normal human impacted third molars were surgically removed and collected. Dental follicle tissues were digested in a solution of 0.1 U/ml collagenase and 1 U/ml dispase (Roche, Mannheim, Germany) for 1 h at 37 °C. Attached hDFCs were cultured at 37 °C in 100-mm dishes using MSC growth medium (GM; consisting of MSC basal medium supplemented with fetal bovine serum, L-glutamine and penicillin/streptomycin; Lonza, Walkersville, MD) in a humidified incubator (CO₂ incubator MCO-175M; Sanyo, Osaka, Japan) in the presence of 5% CO₂ in air at 37 °C. Experiments using hDFCs were performed according to the guidelines established by the ethics committee of Nihon University School of Dentistry at Matsudo (Recognition number: 10-036).

Osteogenic differentiation

For induction of osteogenic differentiation, hDFCs from the 5th to 6th passage were seeded at 3.1 × 10⁶ cells/cm² in GM. After 24 h (day 0), media were replaced with MSC osteoinduction medium without DEX (OIM; consisting of osteogenic basal medium supplemented with fetal bovine serum, L-glutamine, penicillin/streptomycin, ascorbate and β-glycerophosphate; Lonza). On day 1 and every 3 days thereafter, medium was replaced with fresh medium.

Total RNA isolation

Total RNA from hDFCs was isolated using miRNeasy Mini Kits (QIAGEN, Valencia, CA) according to the manufacturer’s instructions.

Microarray analysis

Gene expression profiling was performed using SurePrint G3 Human GE microarray 8 × 60 k (Agilent, Santa Clara, CA) according to the manufacturer’s protocol. Briefly, cRNA labeled with Gy3 was generated from 100 ng of total RNA using Low Input Quick Amp Labeling kit, one-color (Agilent), and was fragmented and hybridized to the array using a Gene Expression Hybridization kit. The array was scanned (Agilent DNA Microarray Scanner), and raw data from the microarray was loaded into Gene Spring GX software (Version 11.5; Agilent). Data were normalized according to the manufacturer’s protocols. Regulated genes were recognized as having more than a two-fold change in OIM culture when compared with GM culture on culture day 17.

RT-PCR and real-time PCR

cDNA was synthesized using a GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA). The PCR mixture (20 µl) containing DyNAmo SYBR Green qPCR kit (Finnzymes, Espoo, Finland), 20 pmol forward and reverse primers and 2 µl cDNA was subjected to amplification with a DNA Engine Opticon 1 (BioRad, Hercules, CA). RT-PCR under the following conditions; preheating at 95°C for 10 min, followed by 27 cycles of 94 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s. PCR fragments were electrophoresed on a 1.5 % agarose gel and subsequently stained with ethidium bromide. For real-time PCR, amplicons were directly detected by measuring the increase in fluorescence caused by binding of SYBR Green using a DNA Engine Opticon 1, with pre-heating at 95 °C for 10 min, followed by 40 cycles as follows: 94°C for 15 s, 57 °C for 30 s, and 72°C for 30 s. Gene expression levels were calculated using the ΔΔCt method. Primers used for RT-PCR and real-time PCR analysis were as follows: 5'-GACCTTTCATGTGCTTTACC-3’ (forward primer for Sema 7A); 5'-GATCTCCATGATTTGAGG-3’ (reverse primer for Sema 7A); 5’-GTCAACGTGATGTTCTCCTT-3’ (forward primer for Plexin C1); 5’-CCTTCTTGCACTTTTACACC-3’ (reverse primer for Plexin C1); 5’-TCCTCTATTGCTCACCTT-3’ (forward primer for β1 integrin); 5’-ACTGCTGACTTGGGATCAG-3’ (reverse primer for β1 integrin); 5’-ATCACATCCTCTCAGGAG-3’ (forward primer for GAPDH); and 5’-ATCGACTGTGGTCTAGG-3’ (reverse primer for GAPDH).

Statistics

Data are expressed as means ± SD of three samples. Significance of differences between culture samples was determined using Student’s t-test and a value of p<0.005 was considered to be statistically significant.

Results

Microarray analysis

We performed microarray analysis to determine the genes regulated in hDFCs by osteogenic induction. The expression of 42,545 genes on the SurePrint G3 Human GE microarray 8 × 60 k were compared between hDFCs cultures with GM and that with OIM on the day 17. We used a two-fold change in the expression density as the cut-off point to determine differentiation. A total of 3,988 genes showed differences in expression in culture with OIM compared to with GM; 2,174 were up-regulated and 1,814 were down-regulated (Fig. 1).

Recently, increasing evidence has pointed to a role for semaphorins during bone development, as well as in the control of normal bone homeostasis. Expression of several semaphorins was up-regulated in hDFCs by osteogenic induction (Table 1). Among these, semaphorin 7A was the most up-regulated in hDFCs cultured with OIM as compared to GM. Sema3C, Sema4D and Sema6D were slightly up-regulated in OIM culture, although the expression of Sema4D and Sema6D was weak in hDFCs. In contrast, Sema3 group members other than Sema3C were down-regulated in OIM culture.

Expression of semaphorin 7A during osteogenic differentiation

Because Sema 7A was the most up-regulated among
Table 1. Gene Expressions of Semaphorin Family Members

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Fold (OIM/GM)</th>
<th>GM culture Raw intensity</th>
<th>Flag</th>
<th>OIM culture Raw intensity</th>
<th>Flag</th>
<th>Genebank ID</th>
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<tr>
<td>Sema3A</td>
<td>0.5</td>
<td>362.3</td>
<td>Detected</td>
<td>198.6</td>
<td>Detected</td>
<td>NM_006080</td>
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<td>Sema3B</td>
<td>0.2</td>
<td>5652.8</td>
<td>Detected</td>
<td>1054.2</td>
<td>Detected</td>
<td>NM_004636</td>
</tr>
<tr>
<td>Sema3C</td>
<td>2.3</td>
<td>253.1</td>
<td>Detected</td>
<td>586.6</td>
<td>Detected</td>
<td>NM_006379</td>
</tr>
<tr>
<td>Sema3D</td>
<td>0.1</td>
<td>898.1</td>
<td>Detected</td>
<td>102.8</td>
<td>Detected</td>
<td>NM_152754</td>
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<tr>
<td>Sema3E</td>
<td>0.2</td>
<td>18.9</td>
<td>Detected</td>
<td>2.9</td>
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<td>NM_012431</td>
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<tr>
<td>Sema3F</td>
<td>0.5</td>
<td>131.6</td>
<td>Detected</td>
<td>68.6</td>
<td>Detected</td>
<td>NM_004186</td>
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<tr>
<td>Sema3G</td>
<td>0.6</td>
<td>3.8</td>
<td>Not Detected</td>
<td>2.1</td>
<td>Not Detected</td>
<td>NM_020163</td>
</tr>
<tr>
<td>Sema4A</td>
<td>1.1</td>
<td>86.9</td>
<td>Detected</td>
<td>97.8</td>
<td>Detected</td>
<td>NM_022367</td>
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<tr>
<td>Sema4B</td>
<td>1.4</td>
<td>116.6</td>
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<td>159.5</td>
<td>Detected</td>
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<tr>
<td>Sema4C</td>
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<td>3196.4</td>
<td>Detected</td>
<td>5360.7</td>
<td>Detected</td>
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<td>Sema4D</td>
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<td>Detected</td>
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<td>Sema4F</td>
<td>0.8</td>
<td>103.9</td>
<td>Detected</td>
<td>83.2</td>
<td>Detected</td>
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<tr>
<td>Sema4G</td>
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<td>XR_428710</td>
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<td>Sema5A</td>
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<td>Detected</td>
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<td>NM_001256346</td>
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<tr>
<td>Sema5B</td>
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<td>2.8</td>
<td>Not Detected</td>
<td>NM_001031702</td>
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<td>Sema6A</td>
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<td>38.2</td>
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<td>46.9</td>
<td>Detected</td>
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<td>147.0</td>
<td>Detected</td>
<td>137.0</td>
<td>Detected</td>
<td>NM_032108</td>
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<tr>
<td>Sema6C</td>
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<td>Detected</td>
<td>517.8</td>
<td>Detected</td>
<td>NM_001178061</td>
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<td>Sema6C</td>
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<td>Detected</td>
<td>81.5</td>
<td>Detected</td>
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<tr>
<td>Sema6D</td>
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<td>8.3</td>
<td>Not Detected</td>
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<td>Detected</td>
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<tr>
<td>Sema7A</td>
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<td>34.2</td>
<td>Detected</td>
<td>456.5</td>
<td>Detected</td>
<td>NM_003612</td>
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</tbody>
</table>

Figure 1. Scatter plots for gene expressions in hDFCs were created with GeneSpring software. Gene expression levels of 42,545 genes were compared between GM culture and OIM culture on day 17. Regulated genes were recognized as having more than a two-fold change.

Semaphorin family members by osteogenic induction using microarray, we next confirmed the gene expression of Sema 7A in hDFC during osteogenic differentiation using RT-PCR and Real time-PCR. As shown in Fig. 2, there was increased mRNA levels for Sema 7A in OIM culture when compared to GM culture on day 17, although no differences were seen between OIM and GM on day 3 using RT-PCR. We then confirmed Sema 7A gene expression in three hDFC samples obtained from three donors during osteogenic differentiation. Sema 7A was significantly up-regulated by OIM in comparison to GM on day 17 in three hDFC
Figure 2. Sema7A mRNA levels in hDFC using RT-PCR. hDFCs were cultured in growth medium (GM) or osteogenic induction medium (OIM).

Figure 3. Gene expression of Sema 7A in hDFCs during osteogenic induction using real-time PCR. Cells were cultured with each medium for the indicated time period. hDFCs were cultured in growth medium (GM: ■) or osteogenic induction medium (OIM: ○). Values represent means ± SD of the results (n=4). *P<0.005; OIM vs GM at indicated time point, analyzed by using Student’s t-test.

Figure 4. Gene expression of PlexinC1 and β1-integrin in hDFCs during osteogenic induction using real-time PCR. Cells were cultured with each medium for the indicated time period. hDFCs were cultured in growth medium (GM: ■) or osteogenic induction medium (OIM: ○). Values represent means ± SD of the results (n=4). *P<0.005; OIM vs GM at indicated time point, analyzed by using Student’s t-test.

Expression of semaphorin 7A receptors during osteogenic differentiation

We next evaluated the gene expression of the semaphorin receptors plexcin C1 and β1-integrin. Expression of plexcin C1 increased on culture with OIM when compared with GM on day 17. In contrast, β1-integrin showed little increase on culture with OIM when compared to GM culture on day 17.

Discussion

Several semaphorins have recently been suggested to be involved in the development and homeostasis of bone. However, the mechanisms and actions of semaphorins are typically investigated by histological study using mouse models, and it is unclear to what extent semaphorins and their signaling pathways are conserved between mice and humans. In this study, we analyzed the gene expression profile in hDFCs between GM culture and OIM culture on day 17, when hDFCs cultured with OIM are in the mineralization stage. We found that several semaphorin family members were up-regulated in hDFCs by OIM. Sema 7A was the most up-regulated gene among semaphorin family members in hDFCs (Table 1). Previous reports have also confirmed the expression of Sema 7A mRNA in mouse osteoblast cell lines and in primary calvarial osteoblasts.

We confirmed the gene expression of Sema 7A in hDFCs using RT-PCR and real-time PCR. The gene expression of Sema 7A was elevated in hDFCs cultured with OIM when compared to GM on day 17 in three hDFC samples from three donors (Fig. 3). In addition, the gene expression of Sema 7A is down-regulated on day 3 (confluent stage) and on day 10 (early mineralization stage), as compared to day 0 (proliferation/non-differentiation stage) in all hDFC samples. The MC3T3 cell line and osteoblasts isolated from mouse calvaria expressed Sema 7A mRNA throughout the culture period from the proliferation stage to the nodule mineralization stage. In addition, Sema 7A was significantly decreased during the early nodule stage, and then increased again in the mature mineralized nodule stage in. Sema 7A mRNA thus has a bimodal expression during osteoblast differentiation.

Recently, it has been investigated whether Sema 7A is associated with cell growth, as Sema 7A knockdown cells showed decreased cellular proliferation via cell-cycle arrest at the G1/S phase. On the other hand, Sema 7A was suggested to be a mesenchymal stem cell marker, as it showed significantly increased expression in mesenchymal stem cell culture when compared to non-stem cell mesenchymal cell culture. We therefore believe...
that Sema 7A is associated with cell growth of mesenchymal stem cells/osteogenic lineage cells, including hDFCs, on culture day 0.

Sema 7A also plays an important role in the differentiation and attraction of osteoclasts. Expression of Sema 7A was low at the start of osteoclast culture from spleen-derived cells, and was high during immature osteoclast fusion^{17,26}. Sema7A increases cell fusion, as exposure to exogenous Sema 7A during differentiation enhanced the number of nuclei per osteoclast and reduced the number of individual osteoclasts^{17}. It has also been suggested that Sema 7A triggers osteoclast formation. The increase in Sema 7A expression by osteoblasts in the mature mineralization stage may be related to the induction of osteoclast differentiation.

The gene expression of plexin C1 and β1-integrin, which are two putative Sema 7A receptors, were examined in hDFCs during osteogenic induction. The expression of plexin C1 was significantly increased in hDFCs cultured with OIM when compared with GM on day 17, whereas b1-integrin was slightly increased on culture in OIM. Plexin C1 belongs to the plexin family of cell surface receptors. Plexin C1 was expressed in both MC3T3-E1 and calvarial osteoblasts at all stages during osteoblast differentiation^{17}. The plexin C1 cytoplasmic domain contains a Rho-GTPase binding domain region flanked by two GAP domains, which show RasGAP activity in vitro^{29,30}. However, the contribution of Sema-7A-plexin C1 interactions to osteoblast differentiation is largely unknown.

β1-integrin was slightly increased in hDFCs cultured with OIM when compared with GM at all stages during osteogenic differentiation. An interaction between Sema 7A and b1-integrin was predicted based on the presence of an arginine-glycine-aspartate amino acid sequence, known as an RGD-motif, in the Sema 7A sema domain^{31}. Integrin are heterodimers composed of the α- and β-subunits. In integral membrane proteins, interaction between avb1-integrin and Sema 7A produces IL-10^{31}. Sema 7A initiates T-cell-mediated inflammatory responses through α1β1-integrin via FAK and MAPK activation^{32}. When MC3T3 cells were treated with Sema-7A, ERK-1 and ERK-2 phosphorylation levels increased transiently^{17}. This suggests that Sema-7A is involved in osteoblast migration through the MAPK pathway^{17}. However a-subunit involvement remains to be confirmed in osteoblasts.

In addition, an association study among postmenopausal Korean women associated Sema 7A poly-morphisms with decreased bone mineral density and increased vertebral fracture risk^{13}. Malfunction of Sema 7A might favor osteoclast clustering and increased bone resorption, resulting in osteoporosis.

In this study, we showed that expression of Sema 7A and its receptors was elevated in hDFCs by osteogenic induction. Based on these results, Sema 7A and its receptors may regulate the migration and differentiation of osteoblasts and osteoclasts, and play important roles in bone development and homeostasis.

Conflict of Interest

The author declares no potential conflicts of interest with respect to the authorship and/or publication of this article.

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