氏　　名	翁　瑶
授与した学位	博　士
専攻分野の名称	歯　学
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学位授与の要件	医歯薬学総合研究科機能再生･再建科学専攻 （学位規則第４条第１項該当）
学位論文の題目	O-GlcNAcylation drives calcium signaling towards osteoblast differentiation: a bioinformatics-oriented study (O-GlcNAcylation 型糖鎖修飾は，細胞内カルシウムシグナリングと連動して骨芽細胞の分化を制御する)
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学位論文内容の要旨

Objective: The O-linked β-*N*-acetylglucosaminylation (O-GlcNAcylation) is a protein post-translational modification. O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) are responsible for addition and removal of O-linked β-N-acetylglucosamine (O-GlcNAc) moieties of proteins, respectively. I investigated the correlation of the change in the O-GlcNAcylation pattern with the expression of bone markers as well as the relationship between O-GlcNAcylation and intracellular calcium levels.

Methods: OSMI-1 (OGT inhibitor) and Thiamet-G (OGA inhibitor) were used for suppressing and promoting O-GlcNAcylation, respectively. To examine the role of O-GlcNAcylation levels on osteoblast differentiation, MC3T3-E1 osteoblastic cells treated with OSMI-1 or Thiamet-G were subjected to ALP activity assay and ALP and Alizarin red staining. Correlations between the levels of O-GlcNAcylation and the expression of osteogenic markers as well as OGT were evaluated by qPCR and western blotting. The O-GlcNAcylated proteins, detected by a pan-specific anti-O-GlcNAc antibody, assumed to correlate with Runx2 expression were determined using Pearson's and Spearman's correlation test and retrieved from several databases retrieved then used for further bioinformatics analysis. Intracellular calcium ($[Ca^{2+}]_i$) was monitored in MC3T3-E1 cells treated with OGT and OGA inhibitors using a confocal laser-scanning microscope (CLS). To investigate the influence of $[Ca^{2+}]_i$ changes and calcium signal on the OSMI-1/Thiamet-G-mediated changes in the expression of Runx2, Bsp, OCN, and OGT via an orthogonal experimental design. In this study, a wide variety of reagents related to intracellular calcium movement were used. These were: EGTA, a membrane-impermeable calcium chelator; BAPTA-AM, a membrane-permeable $[Ca^{2+}]_i$ chelator; A23187, a calcium ionophore; and W7, a calmodulin antagonist. The interaction effect between O-

GlcNAcylation and $[Ca^{2+}]_i$ on osteogenic marker expression was determined using stable OGT knockdown MC3T3-E1 cells.

Results: OSMI-1 (10 μM) and Thiamet-G (100 μM) inhibited and enhanced the osteoblast differentiation of MC3T3-E1 cells, respectively. OSMI-1 treatment decreased

ALP activity, the intensity of ALP staining, and calcium deposition in MC3T3-E1 cells, whereas Thiamet-G treatment increased them. OGT expression was negatively controlled by the feedback regulation on changes in O-GlcNAc levels. OSMI-1 clearly decreased the mean relative density of the total O-GlcNAcylation but increased both mRNA and protein expression of OGT; while Thiamet-G increased the O-GlcNAc level but decreased both mRNA and protein expression of OGT. The O-GlcNAcylated proteins with different molecular weights were changed heterogeneously along with the osteoblast differentiation. Seven bands (218, 152, 117, 96, 75, 59, and 30 kDa) were clearly detected by a pan-specific anti-O-GlcNAc antibody during osteoblast differentiation. The time-course profile of global O-GlcNAcylated proteins showed a distinctive pattern with different molecular weights during osteoblast differentiation. O-GlcNAc distribution contributes to the switching from the early to late stages of osteoblast differentiation. The protein expression of Runx2 was significantly correlated with 152, 117, 75, and 30-kDa O-GlcNAcylated proteins in the DMSO, OSMI-1, and Thiamet-G groups using Pearson's and Spearman's correlation test. Based on a literature mining, 21 Runx2-related O-GlcNAcylated proteins were identified as being related to osteoblast differentiation. Bioinformatic analysis revealed that some calcium-related annotations were significantly enriched in the retrieved protein data. "Calcium" was also a high-frequency word from all related GO terms and UniProt Keywords. In the functional enrichment analysis, "GO:0019722_calcium-mediated signaling" and "GO:0071277_cellular response to calcium ion" were related to $[Ca^{2+}]_i$ with a considerable "List Hits" percentage. Treatment with OSMI-1 and Thiamet-G rapidly decreased and increased the $[Ca^{2+}]_i$ in MC3T3-E1 cells, respectively. From the orthogonal designed experiments, OSMI-1 and Thiamet-G significantly influenced the mRNA expression of osteogenic markers (Runx2, Bsp, and OCN). Changes of $[Ca^{2+}]_i$ also significantly influenced the mRNA expression of osteogenic markers. Significant interaction effect between the changes of $[Ca^{2+}]_i$ and O-GlcNAcylation was detected on Runx2, Bsp, and OCN. The interaction effect between the changes in O-GlcNAcylation and $[Ca^{2+}]_i$ was further examined using the stable OGT knockdown cells. $[Ca^{2+}]_i$ changes induced by EGTA, BAPTA-AM, or A23187 significantly changed the mRNA expression of Runx2, Bsp, OCN in the control group; however, these $[Ca^{2+}]_i$-induced changes were interrupted by OGT knockdown for the expression of Runx2, Bsp, and OCN.

Conclusion: These findings suggested that O-GlcNAcylation interacts with $[Ca^{2+}]_i$ and elicits osteoblast differentiation by regulating the expression of osteogenic markers. The literature-mining results also provided a lot of informative hints for future work. Testing its deduction will improve our understanding of the exact mechanism underlying osteoblast differentiation.

論文審査結果の要旨

Objective: Protein posttranslational modification by glycosylation plays an important role in regulating many cellular processes. O-GlcNAcylation is a form of glycosylation. O-GlcNAc transferase (OGT) adds and O-GlcNAcase (OGA) removes GlcNAc, respectively, to serine or threonine residues of nuclear and cytoplasmic proteins through UDP-GlcNAc. The relationship between O-GlcNAcylation and bone metabolism is gaining attention in bone research field. Many previous studies have already reported the O-GlcNAcylation changes during osteoblast differentiation. However, the detailed time-course pattern and distribution of O-GlcNAcylation on various cellular proteins have not been elucidated. This study aimed to reveal the possible mechanisms by which O-GlcNAcylation regulates osteoblast differentiation using a series of bioinformatics-oriented experiments.

Materials and Methods: To examine the role of O-GlcNAcylation in osteoblast differentiation, ALP activity assay, ALP staining and Alizarin red staining were performed in preosteoblastic MC3T3-E1 cells treated with OGT and OGA inhibitors. Correlations between the levels of O-GlcNAcylation and the expression of osteogenic markers as well as OGT were evaluated by qPCR and Western blotting. The O-GlcNAcylated proteins that correlated with Runx2 expression were retrieved from several public databases and used for bioinformatics analysis. Intracellular calcium ($[Ca^{2+}]_i$) was monitored in the cells treated with OGT and OGA inhibitors using a confocal laser-scanning microscope (CLS). The interaction effect between O-GlcNAcylation and $[Ca^{2+}]_i$ on osteogenic marker expression was determined using stable OGT knockdown MC3T3-E1 cells.

Results: Osteoblast differentiation was positively and negatively regulated by the alterlation of O-GlcNAcylation. The time-course profile of global O-GlcNAcylated proteins showed a distinctive pattern with different molecular weights during osteoblast differentiation. The expression pattern of several O-GlcNAcylated proteins was significantly correlated with protein expression of Runx2. Bioinformatic analysis of the retrieved Runx2-related-O-GlcNAcylated-proteins revealed the correlation of calcium signaling. CLS showed that $[Ca^{2+}]_i$ was rapidly changed by alteration of O-GlcNAcylation in MC3T3-E1 cells. O-GlcNAcylation and $[Ca^{2+}]_i$ showed an interaction effect on the expression of osteogenic markers. OGT knockdown disrupted the $[Ca^{2+}]_i$-induced expression changes of osteogenic markers.

Conclusion: The presented study suggested that the protein O-GlcNAcylation plays a complicated role in osteoblast differentiation through the interaction with calcium signaling pathway.

This article, “O-GlcNAcylation drives calcium signaling towards osteoblast differentiation: a bioinformatics-oriented study” (DOI: 10.1002/biof.1774), has been already published in the BioFactors after the international peer-review. Therefore, the thesis defense committee hereby accepts this article as a doctoral dissertation in dentistry.