論文の内容の要旨

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論文題目　Study of Novel Osteoclastogenic Regulators
（新規破骨細胞分化制御因子群に関する研究）

1. Introduction

Bone resorption process is critical to define bone remodeling and to allow constant renewal of the bone matrix. Osteoclasts are unique cells that are responsible for bone resorption by removing mineralized bone matrix and by breaking up the organic bone. Osteoclasts are differentiated from hematopoietic stem cells like macrophages. However, unlike macrophage, they are maturated to multinucleated cells by fusion of mononuclear precursors. These multinucleation processes are necessary required for osteoclastic maturation to undergo efficient bone resorption as mononucleated osteoclasts are unable to resorb bone matrix. However, it remains still uncovered how to maintain and how to coordinately regulate genes in the multinuclei.

Osteoclastogenesis is highly regulated processes requiring multiple stages and is governed by diverse factors. Various soluble factors including RANKL, M-CSF, and steroid hormones have been identified to support such osteoclast differentiation and activity. Similarly, various transcriptional factors including NFκB and AP1 are shown to involve in gene regulations required for osteoclastogenesis. Among them, Nfatc1 is a prime transcriptional factor for osteoclastogenesis. During differentiation stages, Nfatc1 interacts with epigenetic factors to regulate a particular set of target genes at each stage, indicating that distinct epigenetic complexes contribute to the spatiotemporal regulation of each gene expression by Nfatc1 during osteoclastogenesis. However, epigenetic complexes during osteoclastogenesis remain to be identified.

Histone modifications have emerged to be prerequisite for gene regulations by transcriptional factors. Among them, histone methylation is considered as more upstream epigenetic modification to direct cellular differentiation, proliferation, and so on. Similarly, according to previous report that chromatin morphology

within multinucleated osteoclasts exhibits different shape by electron microscope studies, it is most likely that multinuclear gene expression is regulated through epigenetic mechanisms like chromatin remodeling or histone modifications. Nevertheless, there are still little studies to characterize epigenetic status of multinuclei or to identify epigenetic regulators for maintaining multinuclei.

The present study was undertaken A) to uncover the molecular basis to maintain and to regulate multinuclear genes in single osteoclast cell, and B) to identify novel epigenetic regulatory factors supporting Nfatc1 function during the osteoclastogenesis.

2. Results

A. Selective gene expression within multinuclei of mature osteoclasts

Although multinucleation of non-osteoclastic cells are known to cause severe disease, multinucleated osteoclasts are functionally normal and the number of nuclei positively correlates with their bone resorption activity. However, it is minimal understanding about intracellular gene regulation of multinuclei. For that reason, we observed the pattern of multinuclear gene expression.

Firstly, we examined whether all nuclei in a multinucleated osteocalst evenly have transcriptional activity, by expression of several nuclear proteins through immunocytochemistry. Although the tested nuclear receptors were distributed in all nuclei, active transcriptional factors such as RNAPII-Ser2P and Nfatc1 showed selective distribution in only certain nuclei. Similarly, when the primary transcripts of osteoclast-specific genes (Nfatc1, CtsK, and TRAP) and house-keeping gene (βTubulin) were directly detected by RNA FISH, their distribution was limited among multinuclei. These results suggested that all nuclei in a multinucleated osteoclast are not transcriptionally active. We then examined the possibility of their natural diffuse by shuttling among multinuclei by using two cell lines that stably express ZsGreen-NLS and DsRed-NLS proteins individually. When two individual cells were mixed and differentiated to multinucleated osteoclasts, all nuclei were stained by two colors. This result indicated that in osteoclasts, nuclear proteins are transmitted among multinuclei through shared cytoplasm.

Taken together, each nucleus in a multinucleated osteoclast appears to bear selective transcriptional activity.

B. Identification of epigenetic regulators to understand molecular mechanism about selective gene expression within multinuclei.

According to our results, Nfatc1 looks important for gene regulation only in certain nuclei. To explore the molecular basis of such Nfatc1 function during osteoclastogenesis, epigenetic regulators supporting Nfatc1 were searched.

a. Characterization of a novel osteoclastogenic repressor, Jmjd5

Firstly, we tried to identify epigenetic regulators supporting Nfatc1 function by comparing expression pattern during osteoclastogenesis induced by RANKL treatment. Particularly we focused on JmjC-domain-containing proteins which are known as histone demethylase family, because their physiological roles in

bone are totally unknown.

From the expression of ten kinds of JmjC-domain containing proteins, we observed the reduced expression of Jmjd5 during osteoclastogenesis. To verify its functions in the osteoclast differentiation, a stable cell line constitutively expressing shRNAs against Jmjd5 was established, and then osteoclast formation was assayed. Notably, down-regulation of Jmjd5 expression resulted in promotion of osteoclast formation and upregulated expression of osteoclast-specific genes. These results indicated that Jmjd5 is a negative regulator for osteoclast differentiation.

To define a target histone of Jmjd5, various *in vitro* histone demethylase assays[1)] were accomplished. However, we could never detect altered methylation levels on any tested lysine residue of histone H3 by Jmjd5 expression. Instead, we could detect direct interaction between Jmjd5 and Nfatc1 through an *in vitro* binding assay, raising an idea that Nfatc1 is a Jmjd5 substrate. Additionally, when expression of Jmjd5 was down-regulated, the protein level of Nfatc1, but not transcript level, was increased. These results suggested that Jmjd5 regulates the protein stability of Nfatc1.

Jmjd5 is still believed as a histone demethylase due to presence of its JmjC-domain, however the present study has proved that Jmjd5 regulates the protein stability of Nfatc1 by their transient direct interaction.

b. Characterization of a novel co-activator for the Nfatc1, Kiaa1903

Secondly, we tried to biochemically identify novel regulatory components associated with Nfatc1. To purify complexes in similar condition of *in vivo* status, an endogenous-antibody column against Nfatc1 was established and applied for nuclear extracts of Raw264 cells stimulated by RANKL. Among several candidates, an uncharacterized protein, Kiaa1903, was identified as an associating component for the known chromatin remodeler SWI/SNF complex. This protein appeared a novel component of SWI/SNF chromatin remodeler complex because of its SANT domain which is also found in some factors of chromatin remodeler and its association with components of SWI/SNF complex. Indeed, expression of Kiaa1903 was osteoclast-specific among the tested tissues and cells.

To test a co-regulator role of Kiaa1903 for Nfatc1, a luciferase assay was performed with known co-regulators against Nfatc1. The overexpression of Kiaa1903 enhanced the transcriptional activity of Nfatc1. Similarly, when expression of Kiaa1903 was knock-down by shRNA, osteoclast formation was decreased with lowered expression of osteoclast-specific genes. From these results, Kiaa1903 looks to serve as a co-activator for Nfatc1 during osteoclast differentiation. To characterize Kiaa1903 as an epigenetic regulator, a ChIP experiment was performed with histone methylation markers. When expression of Kiaa1903 was down-regulated, the levels of histone H3Lys4 trimethylation on promoters of Nfatc1-target genes were decreased. Also, the complexes of an Nfatc1 containing SWI/SNF complex could not be recruited on their target genes in absence of Kiaa1903.

From these findings, we presume that Kiaa1903 is a novel component of SWI/SNF-type chromatin remodeling complex during osteoclast differentiation.

3. Conclusion

So far, function of various regulators involved in osteoclastogenesis has been characterized by observation of bone phenotype about their knockout mice. However, it is still insufficient to understand osteoclastic differentiation and function at molecular levels. In this study, we detected the intracellular regulation within multinuclei of mature osteoclasts by using various *in vitro* approaches. Also, through biochemical approaches, we identified novel co-regulatory factors required for fine control of Nfatc1 function during osteoclastogenesis.

Our results have revealed that the osteoclasts bear specialized system in order to advantageously maintain their multinuclei. They limit gene expression to only certain nuclei among multinuclei and share necessary nuclear proteins through intracellular shuttling. We presume that by this mechanism osteoclasts reduce the energy required for cellular maintenance and can resorb bone matrix more efficiently.

Although several transcriptional factors such as Nfatc1, NFκB, and AP1 were identified as regulators for normal osteoclast differentiation, epigenetic regulators supporting their function remain to be identified. Present study uncovered an uncharacterized Jmjd5 as a negative regulator for Nfatc1. Any histone demethylase activity was not detectable in Jmjd5 in spite of containing JmjC-domain. Rather, it had nonhistone protein, Nfatc1, as its target protein for protein destabilization. We expect that Jmjd5 functions as a hydroxylase like FIH and Jmjd6 already reported, through which it regulates the stability of Nfatc1 proteins through posttranslational modification like hydroxylation.

Recently, it has been demonstrated that involvement of cell type-specific subunit in polymorphic SWI/SNF complex is important for cellular specification and differentiation. However, this SWI/SNF-type complex was uncharacterized in osteoclast although osteoclasts are very unique differentiated cells. In this study, we purified a novel component of SWI/SNF-type chromatin remodeler, Kiaa1903, which is osteoclast-specifically expressed. Any components of SWI/SNF complex could not be recruited on promoters of Nfatc1-target genes, without Kiaa1903 protein. We expect that Kiaa1903 is a key determiner for osteoclast-specific SWI/SNF complex and is a necessary epigenetic partner of Nfatc1 required throughout osteoclastogenesis from early osteoclastogenesis for chromatin reconfigulation.

From present study, we uncovered new molecular mechanisms required for normal osteoclastogensis. Now, we are trying to confirm that newly identified factors by biochemical skills are significant in osteoclastogenesis and osteoclastic function by preparing the knockout mice.

(Reference)

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