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学位論文題目	MafB boosts osteoclastogenesis: A break-through to the current treatment for Multicentric Carpal Tarsal Osteolysis (多中心性手根骨足根骨融解症（MCTO）の治療を目的とした、破骨細胞における転写因子 MafB の機能解析)

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Abstract of thesis

In this dissertation, the author describes the functional consequences of abnormalities of *Mafb* gene, multiple missense mutations in which are identified in a rare congenital disease, multicentric carpal tarsal osteolysis (MCTO). The summary is as follows:

Backgrounds and purpose:

MCTO is a rare skeletal disease with progressive osteolysis at the carpal and tarsal bones. Autosomal missense mutations in the transactivation domain of transcription factor *MAFB* are known to cause MCTO. Because the transcription factor MafB is highly expressed in osteoclasts, it was hypothesized that the missense mutations in *MAFB* promotes osteoclastogenesis in MCTO. Wild-type MafB was reported to negatively regulate osteoclastogenesis *in vitro*. In addition, missense mutation was shown to decrease the transactivation function of MafB. From these findings, it was previously hypothesized that the mutant MafB protein loses its original function and causes upregulation of osteoclast differentiation in MCTO. However, the function of MafB in osteoclastogenesis *in vivo* and its relation to MCTO has not been experimentally demonstrated. Based on these backgrounds, the author aims at clarifying the function of MafB in osteoclast differentiation and its relevance to MCTO using mouse models.

Materials and Methods:

Myeloid specific *Mafb* knock-out mice (*Mafb*$^{f/f}$*::*LysM-Cre) were used in this study to examine the function of MafB in osteoclasts. The effect of osteoclatogenesis against the bone phenotypes of these mice were examined by measuring the bone density of *Mafb*$^{f/f}$*::*LysM-Cre mice at 8 weeks using micro-CT analysis. In addition, resistance against age-related osteoporosis and RANKL-induced bone loss was also analyzed using micro-CT analysis. Osteoclatogenesis ability of the bone marrow cells of *Mafb*$^{f/f}$*::*LysM-Cre mice was measured by primary osteoclast differentiation assay and pit assay. Moreover, RNA-sequencing was conducted to analyze the candidate pathways that MafB could be regulating during osteoclast differentiation. Following these findings, mouse harboring the human MCTO mutation (*Mafb*$^{MCTO/MCTO}$) was generated using the CRISPR-Cas9 system to relate the author's findings from *Mafb*$^{f/f}$*::*LysM-Cre mice to the pathophysiology of MCTO. Bone phenotype of femurs and tarsal bones was analyzed through micro-CT. To analyze the effect of MCTO on the MafB protein itself, transactivation level of MCTO mutated MafB was measured using luciferase assay against known MafB target promoters. Finally, MCTO mutation was related to osteoclastogenesis ability of *Mafb*$^{MCTO/MCTO}$ mice, by primary osteoclast differentiation assay using bone marrow cells of *Mafb*$^{MCTO/MCTO}$ mice.

Results:

The author first conducted a micro-CT analysis of the femur bone of *Mafb*$^{f/f}$*::*LysM-Cre mice, finding an increase in bone density compared to control, and thus, he hypothesized that MafB promotes osteoclast differentiation *in vivo*. This was further confirmed by *Mafb*$^{f/f}$*::*LysM-Cre mice exhibiting resistance against osteoclast driven bone resorption. Culturing osteoclasts from bone marrow cells of *Mafb*$^{f/f}$*::*LysM-Cre mice showed lower numbers of multinucleated cells compared to control, especially at 42 hours after RANKL-induction. To identify the pathway that MafB regulates in osteoclastogesis, RNA-sequencing was performed against *Mafb*$^{f/f}$*::*LysM-Cre mice derived osteoclasts at 42 hours after RANKL-induction. Osteoclast inhibitory genes and markers of other immune cells including macrophages, were up-regulated in *Mafb*$^{f/f}$*::*LysM-Cre mice derived osteoclasts. From this, it was predicted that MafB physiologically promotes osteolastogenesis by fine tuning osteoclast precursors to the osteoclast linage.

However, the results obtained from *Mafb*$^{f/f}$*::*LysM-Cre mice contradicted to the symptoms of MCTO patients if the MCTO-related *MAFB* missense mutations result in loss-of-function. Therefore, the bone phenotypes of *Mafb*$^{MCTO/MCTO}$ mice were analysed though micro-CT. The bone density of femur bones and tarsal bone volume was comparable between control and *Mafb*$^{MCTO/MCTO}$ mice at 2 weeks. However, both femur bone density and tarsal bone volume were significantly lower in *Mafb*$^{MCTO/MCTO}$ mice by 8 weeks. The function of MCTO mutated MafB were also examined by luciferase assay against reported target genes of MafB, where MCTO mutated MafB increased transcriptional ability compared to MafB containing the intact protein sequence. From the *in vivo* phenotype of *Mafb*$^{MCTO/MCTO}$ mice, it was suggested *Mafb*$^{MCTO/MCTO}$ mice increases osteoclast differentiation. Therefore, osteoclast differentiation was assessed using primary osteoclast differentiation assay using *Mafb*$^{MCTO/MCTO}$ mice derived cells. As a result, *Mafb*$^{MCTO/MCTO}$ mice showed increased multinucleated cell number compared to control, suggesting that MCTO occurs from hyper-differentiation of osteoclasts.

Discussion:

In this study, the author evaluated the function of MafB in osteoclastogenesis *in vivo* for the first time. The results indicated that MafB promotes the differentiation of osteoclasts and that the MCTO mutation activates transcriptional function of MafB. Therefore, the author hypothesized that MCTO is caused by over-differentiating osteoclasts. These

were shown by *Mafb*$^{f/f}$*::*LysM-Cre mice being resistance against osteoclast driven bone resorption and primary osteoclast differentiation experiments showing lower multinucleated osteoclasts. RNA sequencing suggested that MafB regulates osteoclastogenesis at later stages of differentiation by priming osteoclast progenitors to the osteoclast linage. This study demonstrated that wild-type MafB positively regulates osteoclastogenesis at later stages, although previous studies suggested that MafB worked as a negative regulator against osteoclastogenesis.

It was thus predicted that the MCTO missense mutations upregulate the MafB function. The *in vivo* bone phenotypes of *Mafb*$^{MCTO/MCTO}$ mice presented osteolysis-like phenotype. The transactivation function of MafB was shown to be increased in MCTO-mutated MafB. Primary osteoclast differentiation assay using *Mafb*$^{MCTO/MCTO}$ mice-derived cells supported this hypothesis, where multinucleated osteoclast number were increased in cultures of *Mafb*$^{MCTO/MCTO}$ mice derived cells. These results suggest that MCTO is caused by increased MafB function, which abnormally activate osteoclast function.

Despite these findings, possibilities of alternative causes unrelated to osteoclastgenesis still remain. More investigation through histological examination and rescue experiment using bisphosphonate at the timing of onset must be conducted to truly validate the author's hypothesis.

Abstract of assessment result

【Review】

The present study demonstrated that wild-type MafB promotes osteoclastogenesis and that the missense mutations in *MAFB* gene indeed cause MCTO by enhancing osteoclast differentiation through conferrinig an increase upon the MafB function, through animal models. In this regard, this work is highly valuable not only for our better understanding of pathophysiology of a rare disease, MCTO, but also for potentially facilitating the drug development for MCTO patients. This paper deserves the credit for PhD in medical sciences.

【Result】

The final examination committee conducted a meeting as a final examination on 22/01/2019. The applicant provided an overview of dissertation, addressed questions and comments raised during Q&A session. All of the committee members reached a final decision that the applicant has passed the final examination.

【Conclusion】

Therefore, the final examination committee approved that the applicant is qualified to be awarded a Doctor of Philosophy in Human Biology.