# Abstract of Thesis

Name ( BHONGSATIERN PHAN )

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| Title | Analysis of matrix vesicle-mediated mineralization through organelle interactions<br>(オルガネラ相互作用を介した基質小胞性石灰化の解析) |

Mineralization is the deposition of mineral crystals into the extracellular matrix, leading to hard tissue formation, such as bone and tooth. The key mechanism in mineralization is the secretion of matrix vesicles (MVs), which are a special type of extracellular vesicles produced by hard tissue-forming cells. Thus, clarifying the basic molecular mechanism of MV-mediated mineralization is fundamental to understanding the pathogenesis of hard tissue diseases and developing new treatment interventions. Previous studies suggest that MVs are formed in osteoblastic lysosomes. However, how MVs are formed in lysosomes has not been investigated. It is becoming increasingly recognized that cell organelles do not function as autonomous single units but rather in close communication with other organelles by making membrane contact sites. Therefore, the aim of this study is to investigate how MVs are formed through organelle interactions.

The murine osteoblastic cell line, KUSA-A1, was used as a model to study MVs and mineralization as they have uniquely high osteogenic potential and could form mineralized nodules within a few days when cultured in osteogenic media. To analyze osteoblastic lysosomes, lysosomal membrane protein in KUSA-A1 was fused with HA-tag, and a rapid lysosome immunopurification (LysoIP) technique was performed to isolate intact lysosomes. Lysosome enrichment was validated by immunoblot and β-hexosaminidase assay. Whole-cell lysates and isolated lysosomes were obtained from KUSA-A1 cultured in osteogenic media (osteogenic lysosomes). Differential expression analysis of proteomic data of these samples confirmed accumulated lysosome-specific proteins in isolated lysosomes, as compared to whole cell lysates. Mitochondrial proteins were found enriched in osteogenic lysosomes. The results suggest the involvement of lysosome-mitochondria (L-M) interaction in MV-mediated mineralization.

To confirm L-M interaction, we labeled and traced mitochondrial contents using MitoBlue staining. The result demonstrated an increased mitochondrial content transfer to the lysosome in osteogenic-induced cells. Then, the expressions of L-M interaction-related proteins were examined by immunoassay. Proteins responsible for mitochondrial fusion and fission and L-M contact modulators were upregulated, but mitophagy-related proteins were not induced. Furthermore, the imaging analysis also confirmed there were no clear signs of mitophagy or mitochondria-derived vesicles. Then, live cell imaging was applied to observe direct contact between lysosomes and mitochondria. *Lamp1-mNeonGreen* expressing KUSA-A1 was generated to track lysosomes under a fluorescent microscope. Results from live imaging analyses revealed an increased number of lysosome and mitochondria direct contact and longer contact duration were evident in osteogenic-induced cells. Then, a knockout cell line of the *Tbc1d15* gene, which is associated with direct contact formation between lysosomes and mitochondria, was generated

through genome editing. In *Tbc1d15* knockout cells, we observed increased MV accumulation under scanning electron-assisted dielectric microscopy. Consequently, enhanced calcified nodule formation was observed. Therefore, lysosomes and mitochondria interact through direct contact, which is regulated by Tbc1d15, during MV-mediated mineralization.

To further investigate the role of lysosomes during hard tissue formation *in vivo*, *Twist2Cre; Rosa26-lox-stop-lox-Tmem192-3xHA* (*Twist2*-LysoTag) mouse line was established for *in vivo* LysoIP. Lineage tracing results showed *Twist2Cre* targeted osteoblast lineage in mouse calvaria. Lysosomes were successfully isolated from the mouse osteoblasts of *Twist2*-LysoTag mouse calvarias. Electron microscopic observation revealed the presence of electron-dense, vesicle-like structures within the isolated lysosomes, indicative of MVs. Immunoblot and proteomic profiling showed lysosome enrichment in isolated lysosome samples, confirming the validity of our mouse line and method. Proteins related to mineralization were found enriched in calvarial lysosomes, as compared to lung lysosomes which were used as control. The results support the role of lysosomes in the process of bone formation.

In conclusion, direct contact of lysosome and mitochondria in osteoblasts is implicated in MV-mediated mineralization. This finding opens up the possibility of modulating mineralization by targeting organelle functions. Further study on the molecules involved during contact could provide a more complete understanding of the mechanism. Application of *Twist2*-LysoTag mice for *in vivo* LysoIP could be a potential tool for elucidating the remaining mechanisms underlying osteogenesis.

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論文審査の結果の要旨及び担当者

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**論文審査の結果の要旨**

本研究は、硬組織形成に重要な役割を担う基質小胞について、培養骨芽細胞からのリソソーム迅速単離法やプロテオーム解析、イメージング解析を用いて、細胞内における形成過程の詳細を解析したものである。

その結果、基質小胞形成時にはリソソームとミトコンドリアが相互作用すること、またそのメカニズムとしてリソソームとミトコンドリアの直接接触が関与していることが明らかとなった。さらにリソソーム迅速単離による解析が生体硬組織にも応用可能であることが示唆された。

これらの研究成果は、歯槽骨やセメント質の恒常性維持機構や修復機構の理解を深め、歯周組織再生療法の開発につながる基盤情報を提供するものであり、博士（歯学）の学位を授与するのに値するものと認める。