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The University of Osaka

Abstract of Thesis

Name (RUENGSINPINYA LERDLUCK)	
Title	The role of G protein subunit β1 in skeletal development (骨格発生におけるGタンパク質サブユニットβ１の役割)

Abstract of Thesis

Introduction

Intramembranous ossification and endochondral ossification are required for skeletal development. For intramembranous ossification, bone matrices are directly produced and mineralized by osteoblasts. However, endochondral ossification requires cartilage template with chondrocyte differentiation to be replaced by bone. The impairment of ossification results in interference of normal skeletal development and possibly cause disproportionate short stature shown in skeletal dysplasia.

G protein subunit β1 (GNB1/Gβ1) is a component of heterotrimeric G proteins which is significant for signal transduction in various biological events including skeletal development. The mutations of *GNB1* gene in humans cause global developmental delays, neurodevelopmental disability, and persistent growth delays. Furthermore, *Gnb1*-knockout (KO) mice exhibits delayed growth and sometimes manifests neural tube defects with no calvaria formation, suggesting the involvement of GNB1 in skeletal development. Therefore, the purpose of this study was to determine the role of GNB1 in skeletogenesis by analyzing *Gnb1*-KO mice.

Materials and methods

Mice:

The Osaka University Institute Animal Experiment Committee approved all animal studies including *Gnb1*-KO mice with regulatory guidelines.

In situ hybridization (ISH):

The antisense RNA probes labelled with digoxigenin (DIG) for specific-stage chondrocyte differentiation markers and *Gnb1* were used with the alkaline phosphatase-conjugated anti-DIG antibody to detect the signals.

Histology and immunohistochemistry:

Hematoxylin and eosin (H&E), von Kossa, or immunohistochemistry were performed in the tibial paraffin sections. For X-gal staining, after fixation of hindlimbs, they were stained with X-gal and embedded in paraffin. For 5-ethynyl-20-deoxyuridine (EdU) labeling, the EdU labeling solution injected in pregnant mice. Then, detection of EdU-positive cells was performed in tibial paraffin sections from harvested embryos.

Skeletal preparation:

After fixation of the embryos, the staining of bone and cartilage tissues was performed using 0.15% Alcian blue and 0.002% alizarin red.

Primary culture of osteoblasts:

The culture of calvaria derived from primary osteoblasts was grown in osteogenic induction medium with or without treatment with BMP2. To detect the mineralization of bone matrices, alizarin red S staining was performed in cell cultures.

Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR):

Total RNA was harvested from hindlimbs or cultured primary osteoblasts. The cDNA was synthesized after denaturation of the total RNA and used for real-time PCR reaction. The relative mRNA

expression levels were calculated using the comparative CT method.

Statistical analyses:

The unpaired two-tailed Student's *t*-test was used to analyze the comparisons of two groups. The two-way ANOVA followed by Bonferroni *post hoc*-test was used to analyze the comparisons of multiple groups. p values < 0.05 were considered significant.

Results and Discussion

To determine the role of GNB1 in skeletogenesis, I first investigated the expression of *Gnb1* in bone tissues. The X-gal staining and ISH consistently showed that osseous tissue including cartilage and perichondrium highly expressed *Gnb1*, suggesting the involvement of GNB1 in skeletal development. Next, the analysis of the skeletal structure in *Gnb1*-KO mice was performed by whole mount skeletal preparation. The results revealed that *Gnb1*-KO mice manifested dwarfism, shortened limbs and less ossifying area in long bones and vertebra compared with WT littermates. Additionally, some *Gnb1*-KO mice showed no calvaria formation. The results indicated that GNB1 deficiency affects both endochondral and intramembranous ossification.

Second, to investigate the specific stage of endochondral ossification disrupted by *Gnb1* deficiency, I histologically analyzed the tibias in *Gnb1*-KO mice. The H&E staining showed that zone of chondrocyte hypertrophy was decreased in *Gnb1*-KO mice. Consistent with the results of skeletal preparation, tibias of *Gnb1*-KO mice showed less mineralized area in von Kossa staining. Moreover, the analyses of ISH exhibited similar pattern of *Col2a1*, *Runx2*, *Osterix, Ihh* and *Ppr* expression in *Gnb1*-KO and WT littermates. Conversely, *Col10a1* and *Mmp13* expression, the hypertrophic chondrocyte markers in late stage of chondrocyte differentiation, was remarkably decreased in *Gnb1*-KO mice. Therefore, these results suggest a delay in the late stage of endochondral ossification in the absence of *Gnb1* without the association of the expression of *Runx2, Osterix*, *Ihh*, and *Ppr*.

To confirm the results of ISH, immunohistochemistry experiments using Col10 and Mmp13 antibodies showed the similar results to ISH in *Gnb1*-KO mice. Furthermore, the expression of chondrocyte differentiation marker genes was quantitatively examined in bone tissue by RT-qPCR. The *Col10a1* and *Mmp13* mRNA level in *Gnb1*-KO mice was significantly reduced whereas other genes were not affected. These data supported that GNB1 involves in the late stage of endochondral ossification by controlling the expression of *Col10a1* and *Mmp13*.

Next, to examine if the shortening of bone was due to the defective chondrocyte proliferation in *Gnb1*-KO mice, *in vivo* EdU assays were performed. The ratio of proliferating chondrocyte between *Gnb1*-KO and WT littermates was not significantly changed, suggesting that the defect of endochondral ossification in *Gnb1* deficiency was due to the impairment of chondrocyte differentiation.

To investigate the effects of the *Gnb1* deletion to osteoblast differentiation and bone formation, the culture of primary osteoblasts was performed. Alizarin red staining and RT-qPCR analyses of osteoblast specific marker genes, *Col1a1*, *Alp*, and *Ocn,* were intact in *Gnb1*-KO osteoblasts. These results indicated that *Gnb1* seems to be independent in regulation of osteoblast differentiation and activity, and absence of calvaria formation in *Gnb1*-KO mice is mainly resulted from the disruption of cranial neural tube closure because GNB1 is also important for embryonic neurogenesis.

Conclusion

These findings suggest that GNB1 is important and at least involves in the late stage of endochondral ossification by controlling the expression of *Col10a1* and *Mmp13.*

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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、骨格形成の制御過程における G タンパク質関連分子である G protein subunit β1 の役割について解析を行ったものである。

その結果、G protein subunit β1 遺伝子欠損マウスにおいて、内軟骨性骨形成の後期過程が阻害されていることを見出した。一方、G protein subunit β1 遺伝子欠損マウス由来の骨芽細胞の分化および機能は、同腹野生型マウスと比較して、影響を受けていないことを示した。G protein subunit β1 遺伝子欠損マウスで観察された骨格形成障害は、内軟骨性形成の遅延によるものであることを解明し、骨格形成過程における分子メカニズムの一端を明らかにした。

以上の知見は、内軟骨性骨形成の制御機構に対する理解を深め、骨格形成障害疾患の診断あるいは治療法の開発に指針を提示するものであり、博士（歯学）の学位授与に値するものである。