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研究課題名（和文）Elucidation of the molecular mechanisms for the impaired bone formation in disuse osteoporosis and GC-induced osteoporosis using Fkbp5 knockout mice

研究課題名（英文）Elucidation of the molecular mechanisms for the impaired bone formation in disuse osteoporosis and GC-induced osteoporosis using Fkbp5 knockout mice

研究代表者
ＱＩＮ　ＸＩＮ（QIN, Xin）

長崎大学・医歯薬学総合研究科（歯学系）・特任研究員

研究者番号：６０８４６５５９

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研究成果の概要（和文）：Fkbp5を骨芽細胞および骨細胞の除荷誘導分子として同定し、GC処理の有無にかかわらず野生型およびFkbp5ノックアウトマウスの骨芽細胞濃縮画分からRNAを収集し、骨芽細胞マーカー遺伝子の発現を調べた。そして、骨芽細胞と骨細胞の画分を使用してマイクロアレイ分析を行い、遺伝子注釈および経路分析により選択された遺伝子の発現を、リアルタイム逆転写（RT）-PCRによって分析した。

研究成果の学術的意義や社会的意義
この研究の目的は、無負荷状態でのFkbp5の役割を特定することにより、廃用性骨粗鬆症における骨形成障害の分子メカニズムを解明し、GC誘発性骨粗鬆症における骨形成障害の原因となる分子を特定することである。また、 Fkbp5ノックアウトマウスを使用して、GC誘発性骨粗鬆症におけるRunx2タンパク質の減少のメカニズムを明らかにする。 Fkbp5は廃用性骨粗鬆症において新しい機能を持つため、廃用性骨粗鬆症の分子メカニズムを明らかにする珍しい研究である。

研究成果の概要（英文）：We identified Fkbp5 as an unloading-induced molecule in osteoblasts and osteocytes, and collected RNA from the osteoblast-enriched fraction from wild-type and Fkbp5 knockout mice with or without GC treatment, examined osteoblast marker gene expressions. We performed microarray analysis by using osteoblast and osteocyte fractions. The expression of the selected genes by gene annotation and pathway analyses was analyzed by real-time reverse transcription (RT)-PCR.

研究分野：骨代謝科学

キーワード：osteoporosis　bone formation　bone resorption　knockout mice　glucocorticoid　unloading

１．研究開始当初の背景

Osteoporosis is one of the major age-related diseases, which is caused by the imbalance of the activities of osteoblasts (bone formation) and osteoclasts (bone resorption). The major types of osteoporosis in human are postmenopausal osteoporosis, disuse osteoporosis and glucocorticoid (GC)-induced osteoporosis. Fkbp5 is a co-chaperone to attenuate the function of GR by preventing its nuclear translocation. We identified Fkbp5 as an unloading-induced molecule in osteoblasts and osteocytes, and generated Fkbp5 knockout (Fkbp5–/–) mice to reveal the molecular mechanisms of disuse osteoporosis and GC- induced osteoporosis.

２．研究の目的

The first purpose of this study is to elucidate the molecular mechanisms for the impaired bone formation in disuse osteoporosis by identifying the role of Fkbp5 at unloaded condition. The second purpose of this study is to identify the molecules responsible for the impaired bone formation in GC- induced osteoporosis and to clarify the mechanism of the reduction of Runx2 protein in GC-induced osteoporosis using Fkbp5–/–mice. This is an original study, because the role of Fkbp5 in disuse osteoporosis is completely unknown and Fkbp5–/– mouse is an appropriate model to clarify the mechanism for GC-induced osteoporosis.

３．研究の方法

(1) To clarify the molecular mechanism for impaired bone formation in disuse and GC-induced osteoporosis, we will perform microarray analysis by using osteoblast and osteocyte fractions to identify the differentially expressed genes among wild-type and Fkbp5–/– mice with or without tail suspension and with or without GC treatment.

(2) The expression of the selected genes by gene annotation and pathway analyses will be analyzed by real-time reverse transcription (RT)-PCR. We will generate the expression vectors and siRNA of the reproducibly differentially expressed genes, introduce them into primary osteoblasts, and examine osteoblast differentiation by alkaline phosphatase and von Kossa staining. We will also culture them with or without Dex and examine osteoblast differentiation. We will take RNA and protein to detect the expression levels of osteoblast marker genes, including Runx2.

(3) We will generate knockout mice of the finally selected genes by in vitro analysis using CRISPR/CAS9 system, and the phenotypes of the mice with or without tail suspension and with or without GC treatment will be analyzed by histological and micro-CT analyses. The differentiation and functions of osteoblasts will be examined by in situ hybridization, real-time RT-PCR, and Western blot analyses of osteoblast marker genes and proteins. A loxP inserted mouse will also be established if necessary, and we will use 2.3 kb Col1a1 promoter GFP-Cre transgenic mice, which we established, to generate osteoblast-specific knockout mice.

(4) To investigate the relationship of GR, Fkbp5 and Runx2, we will perform immunocytochemistry using GR and Runx2 antibodies to observe their localization in wild-type and Fkbp5–/– primary osteoblasts with or without Dex treatment. Further, we will perform co-IP to examine the binding of GR and Runx2 with or without Dex treatment. Next, we will study how Runx2 protein level is regulated by Dex. First, GR protein level after Dex treatment will be examined. Next, we will examine GR and Runx2 protein stability

in Dex treatment using CHX (protein synthesis inhibitor) and GR and Runx2 ubiquitination by Dex treatment using MG132 (proteasome inhibitor). If GR is not involved in the reduction of Runx2 by Dex treatment, we will find the responsible genes by microarray analysis.

４．研究成果

We identified Fkbp5 as an unloading-induced molecule in osteoblasts and osteocytes. We generated Fkbp5–/– mice. To examine the disuse osteoporosis, tail suspension was used for an animal model for unloading. Previous reports showed that, after tail suspension of C57BL/6 mice for 2 weeks, bone formation is mildly inhibited, bone resorption is markedly enhanced, and bone loss occurs. In our mouse model, in physiological condition, the bone volume of Fkbp5–/– mice was like that of wild-type mice. After tail suspension, bone loss was more severe in Fkbp5–/– mice than wild-type mice in micro-CT analyses (Fig.1). To examine glucocorticoid (GC)-induced osteoporosis, implantation of prednisolone or injection of dexamethasone (Dex) were performed in wild-type and Fkbp5–/– mice. The GC treatment reduced cortical bone volume and bone formation more severely in Fkbp5–/– mice than wild-type mice (Fig.2, 3). These finding indicated that Fkbp5 inhibits bone loss in unloading and GC treatment.

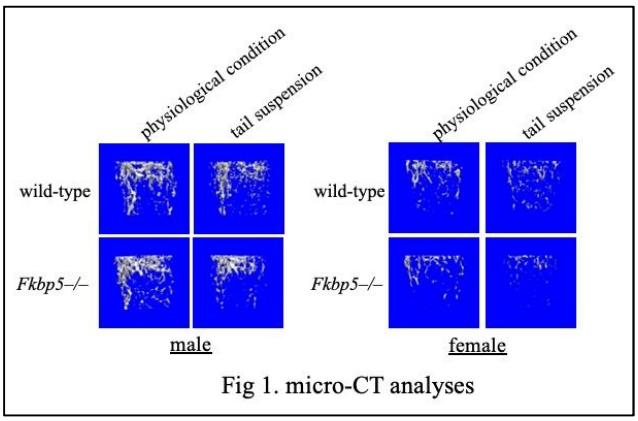

Fig 1. micro-CT analyses

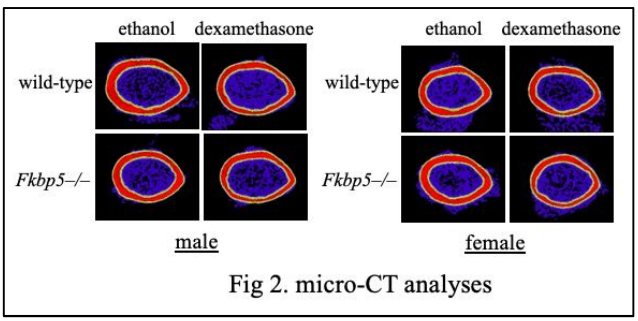

Fig 2. micro-CT analyses

Current understanding of GC-induced osteoporosis is caused by the three ways. Firstly, GC enhanced osteoblasts and osteocytes apoptosis. Several mechanisms of GC-induced apoptosis have been reported, GC increased the pro-apoptotic factors, Bim and Bak, and reduced the pro-survival factor, BclXL in vitro [1, 2]. Excess GC also upregulated p53 protein levels, resulting in increases in p21, Noxa, and Puma [3]. We also observed TUNEL positive terminal hypertrophic chondrocytes, osteoblasts and osteocytes were increased with dexamethasone (Dex) treated mice (data was not shown here).

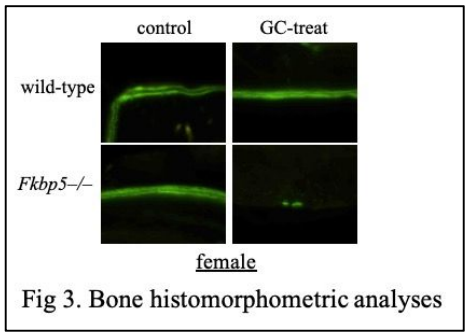

Fig 3. Bone histomorphometric analyses

Secondly, there are some reports showed that GC treatment enhanced osteoclastogenesis and bone resorption in vitro, it has been explained by severely reducing OPG mRNA expression and mildly increasing Rankl mRNA expression, resulting in a marked increase in the RANKL/OPG ratio in stromal cells and osteoblastic cells [4-7]. Excess GCs enhanced bone resorption by extending the life span of osteoclasts [8]. In contrast, we identified Dex inhibit osteoclast number (Trap stain) and osteoclasts function (pit assay) in vitro cell co-culture (data not shown).

Thirdly, GC treatment reduced bone formation by inhibition of osteoblast differentiation. The modulated expression of Wnt antagonists has been extensively examined. Dkk1 was upregulated by Dex in primary cultured human osteoblasts, and Dex induces the reporter activity of the Dkk1 promoter [9, 10].

Furthermore, the suppression of Dkk1 using antisense oligonucleotides or siRNA for Dkk1 abrogates the inhibition of osteoblast differentiation by GCs [11, 12]. Dkk1 expression was upregulated by a prednisolone treatment for 4 weeks [13]. In contrast, the protein expression of Dkk1 is reduced in bone marrow supernatants from wild-type mice after a GC treatment [14]. Thus, the molecular mechanism in the reduction of bone formation of GC-induced osteoporosis is still controversial, thus, requires further study. There is no report on the molecular mechanism of disuse osteoporosis and GC-induced osteoporosis by using Fkbp5–/–mice.

To investigate the molecular mechanisms of GC-induced osteoporosis, we collected RNA from the osteoblast-enriched fraction from wild-type and Fkbp5–/–mice with or without GC treatment. Osteoblast marker gene expressions of *Sp7*, *Col1a1*, *Spp1*, *Bglap2* were drastically reduced, whereas Runx2 mRNA level was not changed (data was not shown here). Therefore, we examined Runx2 protein level, and found that Dex treatment reduces Runx2 protein level to nearly half in wild type mice and more severely in Fkbp5–/–mice. Thus, we realized that Runx2 is regulated at a post-transcriptional level in GC treatment (Fig.4).

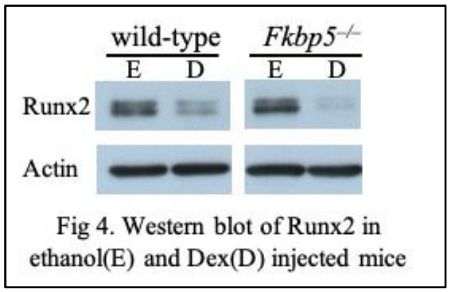

Fig 4. Western blot of Runx2 in ethanol(E) and Dex(D) injected mice

To investigate the relationship of GR, Fkbp5 and Runx2, we performed immunocytochemistry using GR and Runx2 antibodies to observe their localization in wild-type and *Fkbp5*$^{-/-}$ primary osteoblasts with or without Dex treatment. Further, we performed co-IP to examine the binding of GR and Runx2 with or without Dex treatment. We also examined GR and Runx2 protein stability in Dex treatment using CHX (protein synthesis inhibitor) and GR and Runx2 ubiquitination by Dex treatment using MG132 (proteasome inhibitor) (data was not shown here). GR was partly involved in the reduction of Runx2 by Dex treatment. Therefore, we performed microarray analysis to find the responsible genes by using osteoblast and osteocyte fractions, and we identified the differentially expressed genes among wild-type and *Fkbp5*$^{-/-}$ mice with or without GC treatment.

The expression of the selected genes by gene annotation and pathway analyses were analyzed by real-time reverse transcription (RT)-PCR. We will generate the expression vectors and siRNA of the reproducibly differentially expressed genes, introduce them into primary osteoblasts, and examine osteoblast differentiation by alkaline phosphatase and von Kossa staining. We will also culture them with or without Dex and examine osteoblast differentiation. We will take RNA and protein to detect the expression levels of osteoblast marker genes, including Runx2.

We will generate knockout mice of the finally selected genes, and the phenotypes of the mice with or without tail suspension and with or without GC treatment will be analyzed by histological and micro-CT analyses. The differentiation and functions of osteoblasts will be examined by in situ hybridization, real-time RT-PCR, and Western blot analyses of osteoblast marker genes and proteins. A loxP inserted mouse will also be established if necessary, and we will use 2.3 kb Col1a1 promoter GFP-Cre transgenic mice, which we established, to generate osteoblast-specific knockout mice. By combining the results obtained by above experiments, we will publish molecular mechanism for impaired bone formation in disuse and GC-induced osteoporosis will be clarified.

By combining the results obtained by above experiments, molecular mechanism for impaired bone formation in disuse and GC-induced osteoporosis will be clarified.

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５．主な発表論文等

〔雑誌論文〕　計4件（うち査読付論文　4件／うち国際共著　0件／うちオープンアクセス　4件）

１．著者名 Qin Xin、Jiang Qing、Komori Hisato、Sakane Chiharu、Fukuyama Ryo、Matsuo Yuki、Ito Kosei、Miyazaki Toshihiro、Komori Toshihisa	４．巻 1
２．論文標題 Runx2 is required for bone matrix protein gene expression in committed osteoblasts in mice	５．発行年 2021年
３．雑誌名 Journal of Bone and Mineral Research	６．最初と最後の頁 1～1
掲載論文のDOI（デジタルオブジェクト識別子） 10.1002/JBMR.4386	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 －

１．著者名 Qin Xin、Jiang Qing、Nagano Kenichi、Moriishi Takeshi、Miyazaki Toshihiro、Komori Hisato、Ito Kosei、Mark Klaus von der、Sakane Chiharu、Kaneko Hitomi、Komori Toshihisa	４．巻 16
２．論文標題 Runx2 is essential for the transdifferentiation of chondrocytes into osteoblasts	５．発行年 2020年
３．雑誌名 PLOS Genetics	６．最初と最後の頁 1009169～1009169
掲載論文のDOI（デジタルオブジェクト識別子） 10.1371/journal.pgen.1009169	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 －

１．著者名 Jiang Qing、Qin Xin、Yoshida Carolina Andrea、Komori Hisato、Yamana Kei、Ohba Shinsuke、Hojo Hironori、Croix Brad St.、Kawata-Matsuura Viviane K. S.、Komori Toshihisa	４．巻 21
２．論文標題 Antxr1, Which is a Target of Runx2, Regulates Chondrocyte Proliferation and Apoptosis	５．発行年 2020年
３．雑誌名 International Journal of Molecular Sciences	６．最初と最後の頁 2425～2425
掲載論文のDOI（デジタルオブジェクト識別子） 10.3390/ijms21072425	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 －

１．著者名 Moriishi Takeshi、Ozasa Ryosuke、Ishimoto Takuya、Nakano Takayoshi、Hasegawa Tomoka、Miyazaki Toshihiro、Liu Wenguang、Fukuyama Ryo、Wang Yuying、Komori Hisato、Qin Xin、Amizuka Norio、Komori Toshihisa	４．巻 16
２．論文標題 Osteocalcin is necessary for the alignment of apatite crystallites, but not glucose metabolism, testosterone synthesis, or muscle mass	５．発行年 2020年
３．雑誌名 PLOS Genetics	６．最初と最後の頁 1008586～1008586
掲載論文のDOI（デジタルオブジェクト識別子） 10.1371/journal.pgen.1008586	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 －

〔学会発表〕　計0件

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関