様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

科学研究費助成事業　　研究成果報告書

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研究課題名（和文）Elucidation of the functions of Ywhag and Ywhae in bone development using their knockout mice

研究課題名（英文）Elucidation of the functions of Ywhag and Ywhae in bone development using their knockout mice

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研究成果の概要（和文）：Wild-type and Ywhag-/-, Ywhae-/- and Ywhae+/- samples were collected at E15.5, E18.5, 3 weeks and 8 weeks of age. Ywhae-/- embryos showed mild dwarfism, body weight and bone volume of Ywhag-/- mice and Ywhae+/- mice were lower than that of wild-type mice.

研究成果の学術的意義や社会的意義
Elucidation of the molecular mechanisms of 14-3-3s will hopefully provide novel insights into the therapy of many diseases, such as osteoporosis, and is useful for new drugs development for osteoporosis and bone regeneration.

研究成果の概要（英文）：Wild-type and Ywhag-/-, Ywhae-/- samples were collected at E15.5 and E18.5. We stained whole skeletons with alcian blue and alizarin red to identify cartilage and calcified tissues, respectively. Ywhag-/- showed similar level compared with wild-type, while Ywhae-/- embryos showed mild dwarfism, size of Ywhae-/- skeletons was smaller than wild-type.
Due to embryonic lethality of Ywhae-/- mice, wild-type and Ywhag-/-, Ywhae+/- samples were obtained from the mice at 3 weeks and 8 weeks of age. Body weight and bone volume of Ywhag-/- mice and Ywhae+/- mice were lower than that of wild-type mice, especially at 3-6 weeks of age.
The survival rate of Ywhag-/- mice at 8 weeks of age was lower than Ywhag+/+ and Ywhag+/- mice. Survival rate of Ywhae+/- mice was lower than that of wild-type.

研究分野：医歯薬学

キーワード：骨代謝学

様　式　C－１９、F－１９－１、Z－１９、CK－１９（共通）

１．研究開始当初の背景

Runx2 is a transcription factor indispensable for osteoblast differentiation and bone development and homeostasis. Complete absence of bone formation was observed in Runx2 knockout mice. The importance and therapeutic potential of Runx2 in the differentiation of osteobalsts and chondrocytes and bone formation have been proven by many in vivo and in vitro studies.

Our group recently identified a novel Runx2 enhancer, which specifically directs the reporter gene expression to osteoblasts. We identified the genes, which enhanced the osteoblast-specific enhancer, by the screening of cDNA library. Among them, I focused on Ywhag and Ywhae, because their functions in bone development are unknown.

Ywhag and Ywhae belong to 14-3-3 family proteins (14-3-3s), which are critical signaling proteins in cell division, growth, survival, and differentiation, cell cycle regulation, apoptosis, and cell spreading and migration. The involvement of 14-3-3s in bone development is poorly understood, none of reports explains the activation of the Runx2 enhancer reporter construct by Ywhag and Ywhae. Therefore, I planned to examine the functions of Ywhag and Ywhae in bone development by generating *Ywhag*$^{-/-}$ mice and *Ywhae*$^{-/-}$ mice.

２．研究の目的

Runx2 is a major transcription factor for osteoblast differentiation and chondrocyte maturation. A novel Runx2 343-bp enhancer element is sufficient for directing the reporter gene expression to osteoblasts. We found that 14-3-3 gamma (Ywhag) and 14-3-3 epsilon (Ywhae) enhance the activity of Runx2 343-bp enhancer. Therefore, we generated the mice deficient in Ywhag, Ywhae, or both by CRISPR-Cas9 system. The main purpose of this study is to reveal the functions of Ywhag and Ywhae in bone development. This is the first study of 14-3-3s in bone development using knockout mouse models.

３．研究の方法

We performed general analysis of embryos in the differentiation of chondrocytes and osteoblasts, and in the endochondral and intramembranous ossification. Wild-type and KO samples were collected at E15.5, E16.5 and E18.5. We stained whole skeletons with alcian blue and alizarin red to identify cartilage and calcified tissues, respectively. A general histological analysis was done by staining the sections with haematoxylin and eosin to evaluate cell morphology and bone tissue structure. Proliferation was assessed by BrdU incorporation experiments.

We also performed general analysis of growing and adult mice. The samples were obtained from the mice at 3 weeks and 8 weeks of age. In histological analysis, haematoxylin and eosin staining were performed. Osteoblast differentiation was evaluated by immunohistochemistry using Runx2 or Col1a1 antibody. BrdU staining was performed at 3 weeks of age for the evaluation of osteoblast proliferation.

４．研究成果

The F0 mouse with 62 base deletion and the one with 28 base deletion in exon 1 of *Ywhag* were selected, and two *Ywhag*$^{+/-}$ lines were established. The survival rate of *Ywhag*$^{-/-}$ (KO) mice at 8 weeks of age was lower than *Ywhag*$^{+/+}$ (Wt) and *Ywhag*$^{+/-}$ (Ht) mice (Fig. 1). Body weight of *Ywhag*$^{-/-}$ mice was lower than that of wild-type mice at 2-6 weeks of age (Fig. 2). *Ywhag*$^{-/-}$ embryos did not show apparent phenotypes, whereas *Ywhag*$^{-/-}$ mice showed dwarfism at 3

	Line1(229mice)	Line2(124mice)
Ywhag-Wt	93.7% (59/63)	97.1% (33/34)
Ywhag-Ht	88.1% (96/109)	90.3% (56/62)
Ywhag-KO	68.4% (39/57)	71.4% (20/28)
	Line1(99mice)	**Line2(91mice)**
Ywhae-Wt	98.5% (64/65)	96.6% (56/58)
Ywhae-Ht	79.4% (27/34)	78.8% (26/33)
Ywhae-KO	0 (lethal)	0 (lethal)

Fig.1 Survival rate of *Ywhag* KO mice and *Ywhae* KO mice.

weeks of age, and catched up wildtype mice at 8 weeks of age. Histological analysis of *Ywhag*$^{-/-}$ mice at 3 weeks of age showed that cortical bone thickness was reduced, number of osteoblasts which were expressed Runx2 and Col1a1 was drastically reduced, and proliferation was reduced in the layer of growth plate compared with wildtype mice by BrdU labeling (Fig. 3).

To establish *Ywhae*$^{+/-}$ lines, the F0 mouse with 5 base deletion and the one with 31 base deletion were selected. *Ywhae*$^{-/-}$ (KO) embryos showed mild dwarfism, and the mineralization was delayed in skeleton preparation. In histological analysis, the number of BrdU positive cell was reduced in the proliferating layer of the growth plates in *Ywhae*$^{-/-}$ mice compared with wildtype (Wt) mice (Fig.4). *Ywhae*$^{-/-}$ mice died just after birth. Survival rate of *Ywhae*$^{+/-}$ (Ht) mice was lower than that of wildtype (Wt) (Fig. 1).

These findings indicated that 14-3-3 proteins are important for bone development.

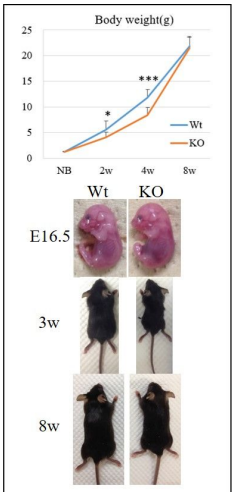

Fig.2 *Ywhag* KO mice.

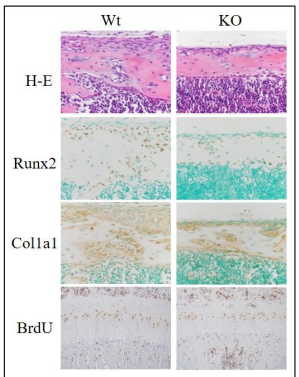

Fig.3 Histological analysis of *Ywhag* KO mice.

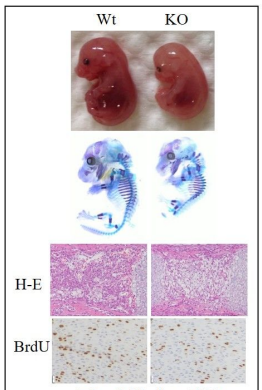

Fig.4 *Ywhae* KO embyo mice.

５．主な発表論文等

〔雑誌論文〕（計　2　件）

① Qin X, Jiang Q, Miyazaki T, Komori T: Runx2 regulates cranial suture closure by inducing hedgehog, Fgf, Wnt, and Pthlh signaling pathway gene expression in suture mesenchymal cells. Hum Mol Genet. 28(6):896-911, 2019. (査読有り)

② Kawane T, Qin X, Jiang Q, Miyazaki T, Komori H, Yoshida CA, Matsuura-Kawata VKDS, Sakane C, Matsuo Y, Nagai K, Maeno T, Date Y, Nishimura R, Komori T: Runx2 is required for the proliferation of osteoblast progenitors and induces proliferation by regulating Fgfr2 and Fgfr3. Sci Rep. 10;8(1):13551, 2018 Sep. doi: 10.1038/s41598-018-31853-0. (査読有り)

〔学会発表〕（計　2　件）

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第36回日本骨代謝学会学術集会シンポジウム，長崎，7月，2018.
Title: Functions of Runx family transcription factors and Cbfb in skeletal development.

② 姜晴，小守壽文．
第122回日本解剖学会総会・全国学術集会シンポジウム，長崎，3月，2017.
Title: Functions of Runx family transcription factors and Cbfb in skeletal development.

〔図書〕（計　0 件）

〔産業財産権〕
○出願状況（計　0　件）

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権利者：
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○取得状況（計　0　件）

名称：
発明者：
権利者：
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取得年：
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〔その他〕
ホームページ等

６．研究組織

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