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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）副作用の少ない小児ステロイド性顎骨骨粗鬆症治療における骨吸収阻害薬の開発

研究課題名（英文）Development for low side effect anti-bone resorption medicines of pediatric steroidal osteoporosis

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交付決定額（研究期間全体）：（直接経費）　　3,600,000円

研究成果の概要（和文）：Bif-1欠損(Bif-1 -/-)マウスでは骨梁の数と骨量が増加している。さらにBif-1 -/-マウスでは破骨細胞数が増加していた。In vivo実験データが示すように、in vitroにおいてBif-1 -/-マウスではRANKL下流のNF-κB やMAPKsシグナルさらに前破骨細胞のRANKの発現、骨吸収活性、破骨細胞の生存率に影響を与えることなく破骨細胞形成が促進した。予想に反して、in vivoで骨形成がBif-1 -/-マウスで上昇していた。In vitroにおいてもBif-1 -/-マウスで骨芽細胞分化と石灰化が亢進していた。

研究成果の学術的意義や社会的意義
Bif-1欠損マウスでは骨梁の数と骨量が増加する。そのメカニズムが解明できたことにより、Bif-1をターゲットにした新たな骨代謝治療薬創出につながる可能性がある。

研究成果の概要（英文）：Bif-1-deficient (Bif-1 -/-) mice showed increased trabecular bone volume and trabecular number. Histological analyses indicated that the osteoclast numbers increased in Bif-1 -/- mice. Consistent with the in vivo results, osteoclastogenesis induced by RANKL was accelerated in Bif-1 -/- mice without affecting RANKL-induced activation of RANK downstream signals, such as NF-κB and MAPKs, CD115/RANK expression in osteoclast precursors, osteoclastic bone-resorbing activity and the survival rate. Unexpectedly, both the bone formation rate and osteoblast surface substantially increased in Bif-1 -/- mice. Osteoblastic differentiation and mineralization were enhanced in Bif-1 -/- mice. Finally, bone marrow cells from Bif-1 -/- mice showed a significantly higher colony-forming efficacy, suggesting that cells from Bif-1 -/- mice had higher clonogenicity and self-renewal activity than those from WT mice.

研究分野：小児歯科学、骨代謝

キーワード：骨代謝　骨芽細胞　破骨細胞　Bif-1　Endophilin B1　SH3GLB1

１．研究開始当初の背景

小児の成長期ではさまざまなホルモンや局所因子が骨のリモデリングを調節し、顎骨をはじめ骨格の成長を促す。近年、喘息などのアレルギー疾患を持つ小児の増加により、ステロイド製剤は必要不可欠となっている。ステロイド製剤は喘息やアレルギー疾患などの治療に効果的である一方、小児患者においては成長障害や骨粗鬆症などの副作用が重大な問題となっている。成人における続発性ステロイド性骨粗鬆症の治療治療には、破骨細胞を標的としたビスホスホネート(BP)製剤が第一選択薬として用いられている。しかし、BP 製剤の長期投与は骨質の低下を引き起こすだけでなく、近年では BP 服用患者に抜歯などの観血的処置を行うことで顎骨壊死を引き起こすことが報告され、歯科界で重大な問題となっている。また、小児に BP 製剤を長期投与すると成長抑制が報告されており、BP 投与の適応症は重篤な骨形成不全症などに限られている。BP 製剤のように破骨細胞を特異的にターゲットとした治療は多くの問題を抱えながらも続発性ステロイド性骨粗鬆症治療に有効である。しかし、成長期の小児においては、生理的な骨の成長に影響しない骨吸収抑制剤の開発が必要である。

２．研究の目的

骨吸収に必須な c-Src の下流で機能する p130Cas 欠損マウスが骨吸収不全による大理石骨病を呈する。本研究では、p130Cas と会合する分子の網羅的解析から新たな骨吸収分子として選別した Bif-1 の生理機能を in vitro および in vivo で解明するとともに、p130Cas と Bif-1 の会合部位を標的とした新たな骨吸収阻害効果をもつ化合物の開発を目指した。

３．研究の方法

①実験動物

The generation of Bif-1−/− mice has previously been described (Takahashi Y et al., 2013). All animal experiments were approved by the Animal Care and Use Committee of Kyushu Dental University (approval number: 16-031).

②組織解析

The tibiae and femurs from wild-type (WT) or Bif-1−/− mice were embedded in a SCEM compound (Section-Lab, Hiroshima, Japan) as previously described (Suzuki N et al., 2016). Five μicrometer of frontal sections of the tibiae were prepared and subsequently stained with tartrate-resistant acid phosphatase (TRAP), a marker enzyme of osteoclasts, and counterstained with methyl green. The $TRAP^+$ multinucleated cells (MNCs) containing more than two nuclei, located on the bone surface were counted as osteoclasts. The bone histomorphometric analyses were performed in the secondary spongiosa of the tibias with an image analyzing software (ImageJ, version 1.51k; NIH, Bethesda, MD), as described previously (Suzuki N et al., 2016).

③破骨細胞分化実験

Bone marrow cells derived from 8-week-old WT or Bif-1−/− mice were cultured for 6 days in the presence of M-CSF (100 ng/mL) and various concentrations of RANKL in α-minimal essential medium (α-MEM) that contained 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin in 96-well plates. After 6 days cultures, cells were fixed with 3.7% formaldehyde in PBS for 10 minutes, and cells stained for TRAP. $TRAP^+$ MNCs containing more than three nuclei were counted as osteoclasts under a microscope.

④プラスミドと遺伝子導入

FLAG-tagged mouse Bif-1 was constructed with standard polymerase chain reaction (PCR) technique using Takara Ex Taq (Takara Bio, Shiga, Japan) in to pcDNA3.1 vector. RAW-D cells, kindly provided from Dr. Kukita (Kyushu University, Japan) (Watanabe T et al., 2014) were transfected with 250 ng per well and 500 ng per well plasmid DNA using 0.75 μL/well and 1.5 μL/well Lipofectamine 3000 (Thermo Fisher Scientific, Foster City, CA), in 48well plates according to the manufacturer's protocol, respectively. Primary osteoblasts were transfected with 50 ng per well and 100 ng per well plasmid DNA using 0.15 μL/well and 0.3 μL per well Lipofectamine 3000, in 96-well plates.

⑤cDNA の合成と遺伝子

Total RNA was isolated from cultured osteoclasts or osteoblasts using the Relia Prep RNA miniprep system (Promega, Madison, WI) according to the manufacturer's protocol, and then complementary DNA was synthesized using Applied Biosystems reverse transcription kit (Thermo Fisher Scientific). Real-time PCR was performed described previously (Murakami A et al., 2017). The primer sequences have been described previously (Murakami A et al., 2017, Hirata-Tsuchiya S et al., 2014).

⑥免疫蛍光染色

Osteoclasts were fixed for 15 minutes with 4% paraformaldehyde in PBS, subsequently blocked for

15minutes with 5% skim milk in PBS at room temperature, and then incubated with the anti-Bif-1 antibody (1:100) for 30 minutes at 37°C. The cells were further incubated with Alexa Fluor 488-conjugated anti-rabbit immunoglobulin G (IgG) (diluted 1:1000; Invitrogen, Carlsbad, CA) for 30 minutes at 37°C. After extensive washes, cells were mounted with Immunon (Lipshaw, Pittsburgh, PA). The subcellular localization of Bif-1 was observed under a fluorescence microscopy (BioRevo, BZ-9000; Keyence, Osaka, Japan).

⑦フローサイトメトリー
Mouse bone marrow cells were cultured in low-cell-binding plates for 4 days. Cells were collected and stained with an Alexa Fluor 488 anti-mouse CD115 antibody (eBioscience, SanDiego, CA) and an anti-RANK antibody (9A725) (phycoerythrin) (Abcam, Cambridge, UK), and analyzed using a FACScalibur flow cytometer with CellQuest software (BD Biosciences).

⑧骨吸収の評価
The bone-resorbing activity of osteoclasts was assessed using an Osteo Assay Plate (Corning, Corning, NY) after 6 days of culture. Cells were removed by the 5% of hypochlorous acid solution. Resorption area was quantified by the ImageJ software as previously described (Chen Y et al., 2016, Dou C et al., 2017).

⑧破骨細胞生存率の測定
After 6 days cultures of osteoclasts, RANKL was removed from the culture medium and further cultured for the indicated periods. The remaining TRAP+ MNCs were counted, and the survival rate is expressed as a percentage of the remaining cells at the initial time point (0 hour).

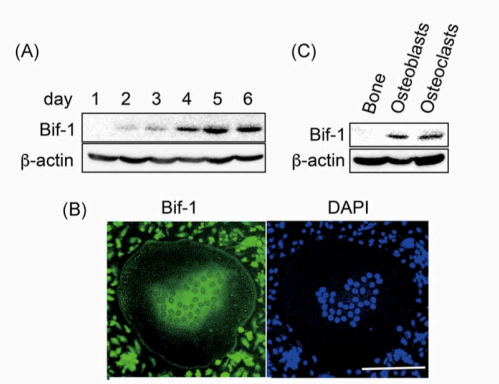
⑨アルカリフォスファターゼ活性と活性染色
For the measurement of alkaline phosphatase (ALP) activity, the cells were fixed with an acetone/ethanol mixture (50:50, vol/vol) and then incubated with an ALP substrate buffer (10 mg/mL p-nitrophenyl phosphate, 0.1 M diethanol amine, and 1 mM MgCl2, pH 8.0). After 15 minutes, adding 5 M NaOH stopped reactions and the absorbance was measured at 405 nm using a microplate reader (Bio-Rad Laboratories, Inc, Hercules, CA). ALP staining was performed as previously described (Katagiri T et al., 1994).

⑩アリザリンレッド染色
The cells were fixed for 10 minutes with 3.7% formaldehyde in PBS and after extensive washes, cells were stained for 30 minutes at room temperature with 1% alizarin red S solution. Alizarin red dye was resolved with 10% formic acid, and the absorbance was measured at 405 nm using a microplate reader.

⑪コロニー形成試験と骨芽細胞分化
Mononuclear cells from WT or Bif-1−/− mice were cultured in six-well plates with α-MEM that contained 10% FBS at a density of 2.0×104 cells per well for 7 days. The cells were further cultured with α-MEM that contained 10% FBS, β-GP (10 mM) and A.A (50 mg/mL) together with or without BMP2 (100 ng/mL) for 7 days. The cells were fixed for 10 minutes with 3.7% formaldehyde in PBS and then stained for ALP. The number of colonies was counted.

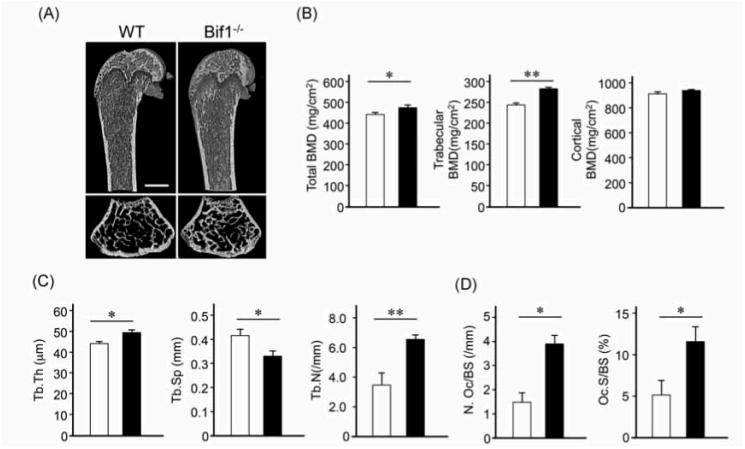
４．研究成果

図１：破骨細胞と骨芽細胞に Bif-1 が発現している



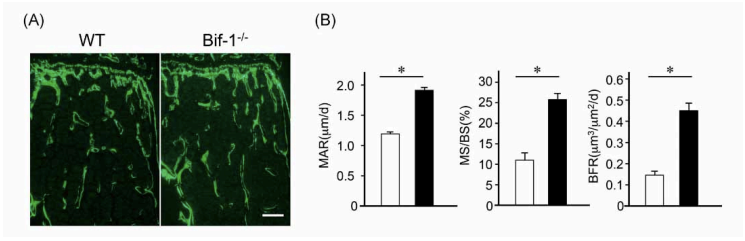
A, Mouse bone marrow cells were cultured with M-CSF for 3 days and then cultured with M-SCF and RANKL for an additional 3 days. Western blot analysis was used to determine the expression levels of Bif-1 during the indicated time for cultures. β-Actin was used as a loading control. Similar results were obtained from three independent experiments. B, Expression of Bif-1 in osteoclasts was immunofluorescence stained for Bif-1. Nuclei were visualized by DAPI. Scale bar = 100μm. C, Western blot analysis was used to determine the expression levels of Bif-1 from bone, osteoblasts and osteoclasts. β-Actin was used as a loading control. Similar results were obtained from three independent experiments.

図２：Bif-1$^{-/-}$ マウスでは破骨細胞の数が増加する



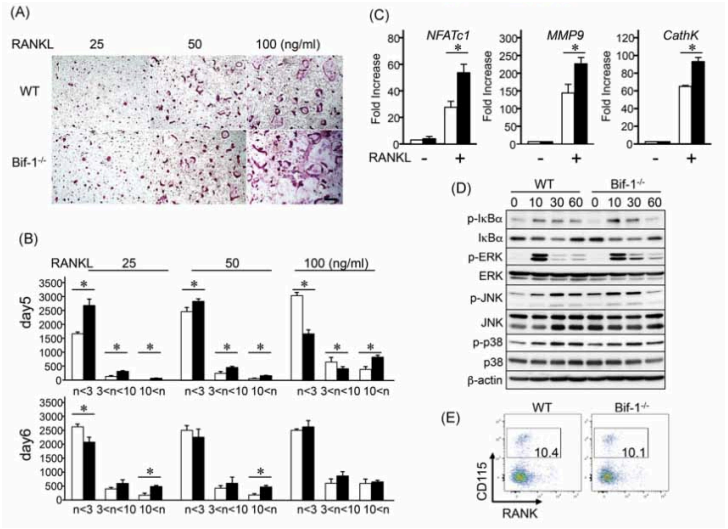
A, Three-dimensional (3D)-μCT images of the proximal metaphyseal regions of tibias of 14-week-old male WT and Bif-1$^{-/-}$ mice. Scale bar = 1mm. B, Total, trabecular and cortical bone mineral density (BMD), (C) trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and trabecular number (Tb.N) of the proximal tibias of 14-week-old male WT (open column) and Bif-1$^{-/-}$ mice (closed column) were measured by dual-energy X-ray absorptiometry (mean ± SD, n = 5). *P<.05, **P<.01. D, Osteoclast number/bone surface (N.Oc/BS) and osteoclast surface/bone surface (Oc.S/BS) of the proximal tibias of 14-week-old male WT (open column) and Bif-1$^{-/-}$ mice (closed column) were measured (mean ± SD, n = 5). *P<.01.

図3：Bif-1$^{-/-}$ マウスでは骨形成が促進している



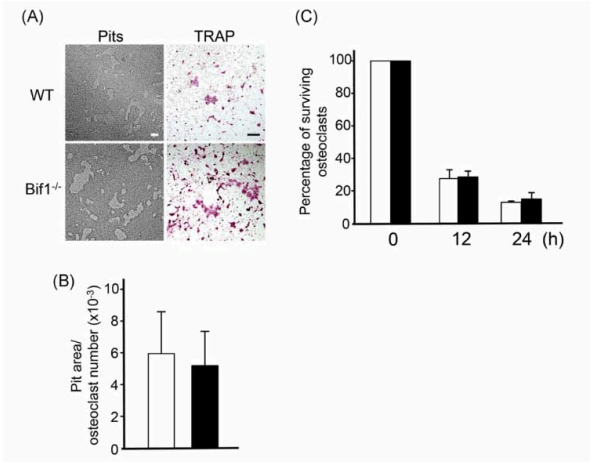
Fourteen-week-old male WT and Bif-1$^{-/-}$ mice were injected with 15 mg/kg calcein twice at an interval of 6 days and euthanized 48 hours after the second injection. A, The calcein double labeling image of tibiae. Scale bar = 100 μm. B, Mineral apposition rate (MAR), mineralized surface/bone surface (MS/BS), and bone formation rate of bone surface reference (BFR) of the proximal tibiae of 14-week-old male WT (open column) and Bif-1$^{-/-}$ (closed column) mice were measured (mean ± SD, n = 5). *P<.01.

図4：Bif-1$^{-/-}$ マウスでは破骨細胞分化が促進する



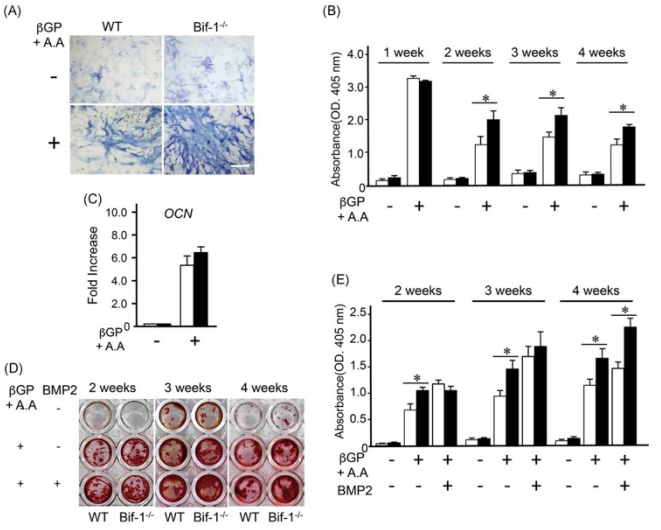
Bif-1$^{-/-}$ mice have an increased osteoclast number. A, Bone marrow cells from WT or Bif-1$^{-/-}$ mice were treated with M-CSF (100 ng/mL) for 3 days and then further cultured with M-CSF and various concentrations of RANKL for 3 days. Cells were stained with TRAP. Scale bar = 100 μm. B, Average number of per TRAP^{+} MNCs nuclei was counted at days 5 and 6. C, Osteoclasts were generated from WT (open column) and Bif-1$^{-/-}$ mice (closed column) mice were treated with M-CSF (100 ng/mL) and RANKL (100 ng/mL) for 6 days. Total RNA was isolated, and NFATc1, matrix metalloproteinase 9 (MMP9), and cathepsin K (CathK), and GAPDH mRNA levels were analyzed using real-time polymerase chain reaction. Data are expressed as the mean ± SD (n = 3). Similar results were obtained from three independent experiments. *P<.01. D, Bone marrow cells from WT or Bif-1$^{-/-}$ mice were treated with M-CSF (100 ng/mL) for 3 days and then treated with RANKL (100 ng/mL) for indicated periods. Western blot analysis was used to determine the expression levels of IκBα, phosphorylated IκBα, phosphorylated ERK, ERK, phosphorylated p38, p38, phosphorylated JNK, and JNK during indicated times for culture. β-Actin was used as a loading control. Similar results were obtained from three independent experiments. E, Bone marrow cells were cultured in the presence of M-CSF for 3 days. Cells stained with anti-RANK-phycoerythrin (PE) and anti-CD115-Alexa 488 antibodies were analyzed using flow cytometry.

図5：Bif-1 は破骨細胞の吸収活性や生存率に影響しない



Bif-1 affects neither the bone resorbing activity nor survival of osteoclasts. A, Bone marrow cells from WT or Bif-1$^{-/-}$ mice were treated with M-CSF (100 ng/mL) for 3 days and then further cultured with M-CSF and RANKL (100 ng/mL) for 3 days on Osteo Assay plates. The resorption pits area was determined using ImageJ software. Cells were stained for TRAP. Scale bar = 100 μm. B, The percentages of resorption areas per TRAP^{+} MNCs on Osteo Assay plates from WT (open column) and Bif-1$^{-/-}$ mice (closed column) were scored (mean ± SD, n = 5). Similar results were obtained from three independent experiments. C, The survival rates of osteoclasts from WT (open column) and Bif-1$^{-/-}$ mice (closed column) after RANKL withdraw were scored (mean ± SD, n = 3). Similar results were obtained from three independent experiments. Bif-1, Bax-interacting factor; M-SCF, macrophage colony-stimulating factor; RANKL, receptor activator of NF-κB ligand; TRAP, tartrate-resistant acid phosphatase; WT, wild-type

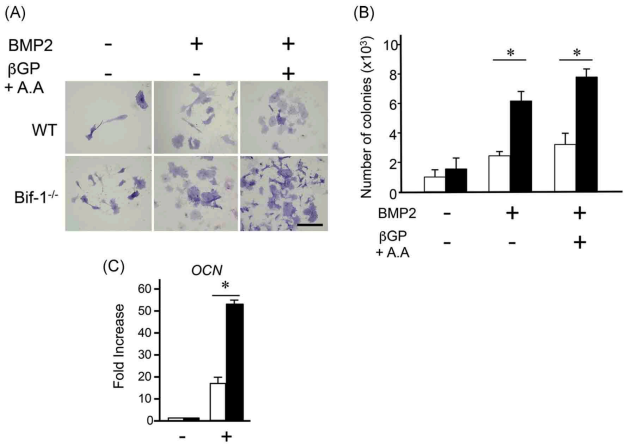
図6：Bif-1$^{-/-}$ マウスでは骨芽細胞分化や石灰化が亢進している



A, Primary osteoblasts from WT and Bif-1$^{-/-}$ mice were treated with β-GP (10 mM) and A.A (50 μg/mL) for indicated periods. The cells were stained for ALP activity after 2 weeks of culture. Scale bar = 100 μm. B, The cells were fixed with an acetone-ethanol mixture and incubated with a substrate solution. ALP activity was subsequently determined. Data are expressed as the mean ± SD (n = 3). Similar results were obtained from three independent experiments. C, Primary osteoblasts from WT and Bif-1$^{-/-}$ mice were treated with β-GP (10 mM) and A.A (50 μg/mL) for 10 days. Total RNA was isolated, and then osteocalcin and GAPDH mRNA levels were analyzed using real-time polymerase chain reaction. Data are expressed as the mean ± SD (n = 3). Similar results were obtained from three independent experiments. D, The cells were stained with alizarin red to determine mineralization. E, Alizarin red dye was extracted with 10% formic acid, and the absorbance at 405 nm was determined with a microplate reader. Data are expressed as the mean ± SD (n = 3). Similar results were obtained from three independent experiments.

図7：Bif-1$^{-/-}$ マウスでは骨髄間葉系幹細胞のセルフリニューアルが亢進している

A, Mononuclear bone marrow adherent cells from WT or Bif-1$^{-/-}$ mice were seeded at a density of 2.0×10^{4} cells per well in six-well plates for 7 days. The cells were further cultured with β-GP (10 mM) and A.A (50 μg/mL) in the presence or absence of BMP2 (100 ng/mL) for 7 days. Cells were fixed with 3.7% formaldehyde in phosphate-buffered saline for 10 minutes and then stained for ALP. Scale bar = 100 μm. B, Number of ALP^{+} colonies was counted. Data are expressed as the mean ± SD (n = 3). Similar results were obtained from three independent experiments. *P<.01. C, Osteoblasts differentiated from bone marrow cells in WT and Bif-1$^{-/-}$ mice were treated with β-GP (10 mM) and A.A (50 μg/mL) for 14 days. Total RNA was isolated, and then osteocalcin and GAPDH mRNA levels were analyzed using real-time polymerase chain reaction. Data are expressed as the mean ± SD (n = 3). Similar results were obtained from three independent experiments. *P<.01. ALP, alkaline phosphatase;



５．主な発表論文等

〔雑誌論文〕　計5件（うち査読付論文　5件／うち国際共著　3件／うちオープンアクセス　2件）

１．著者名 Chaweewannakorn W, Ariyoshi W, Okinaga T, Fujita Y, Maki K, Nishihara T.	４．巻 234
２．論文標題 Ameloblastin attenuates RANKL-mediated osteoclastogenesis by suppressing activation of nuclear factor of activated T-cell cytoplasmic 1 (NFATc1).	５．発行年 2019年
３．雑誌名 J Cell Physiol. 2019	６．最初と最後の頁 1745-1757
掲載論文のDOI（デジタルオブジェクト識別子） 10.1002/jcp.27045.	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 該当する

１．著者名 Ohno K, Fujita Y, Ohno Y, Takeshima T, Maki K	４．巻 -
２．論文標題 The factors related to decreases in masticatory performance and masticatory function until swallowing using gummy jelly in subjects aged 20-79 years.	５．発行年 2020年
３．雑誌名 J Oral Rehabil	６．最初と最後の頁 -
掲載論文のDOI（デジタルオブジェクト識別子） 10.1111/joor.12975	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 Matsubara T, Yaginuma T, Addison WN, Fujita Y, Watanabe K, Yoshioka I, Hikiji H, Maki K, Baron R, Kokabu S	４．巻 132
２．論文標題 Plectin stabilizes microtubules during osteoclastic bone resorption by acting as a scaffold for Src and Pyk2.	５．発行年 2019年
３．雑誌名 Bone	６．最初と最後の頁 115209
掲載論文のDOI（デジタルオブジェクト識別子） doi: 10.1016/j.bone.2019.115209.	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

１．著者名 Touyama K1,2,3, Khan M4, Aoki K4, Matsuda M3, Hiura F3, Takakura N3, Matsubara T1, Harada Y5, Hirohashi Y4, Tamura Y6, Gao J3, Mori K3, Kokabu S1, Yasuda H7, Fujita Y2, Watanabe K2, Takahashi Y8, Maki K2, Jimi E1,	４．巻 120
２．論文標題 Bif-1/Endophilin B1/SH3GLB1 regulates bone homeostasis	５．発行年 2019年
３．雑誌名 J Cell Biochem	６．最初と最後の頁 18793-18804
掲載論文のDOI（デジタルオブジェクト識別子） 10.1002/jcb.29193	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

1．著者名 Takeshima T, Fujita Y, Maki K	4．巻 99
2．論文標題 Factors associated with masticatory performance and swallowing threshold according to dental formula development	5．発行年 2019年
3．雑誌名 Arch Oral Biol	6．最初と最後の頁 51-57
掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/j.archoralbio.2018.12.012	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	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

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