博 士 論 文

Enhanced Bone Anabolic Window with Increased Bone Formation by Abaloparatide Showed Greater Gains in Trabecular and Cortical Bone in Mice: A Comparison with Teriparatide

（アバロパラチドは骨形成促進作用により骨アナボリックウインドウを拡大させ、マウスの海綿骨及び皮質骨を増加させる－テリパラチドとの比較－）

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Abstract

Abaloparatide (ABL) is a novel 34-amino acid peptide analog of parathyroid hormone-related protein. In clinical studies, although ABL showed a greater bone mineral density (BMD) increase than teriparatide (TPTD, human parathyroid hormone 1-34), the responses of ABL to bone formation and resorption markers were weaker, making it difficult to understand the relationship between the bone anabolic window (increase in bone formation versus resorption) and bone mass. In the present study, the effects of ABL and TPTD were compared in mice. Given that the rate of bone turnover is higher in rodents than in humans, the comparison was made with several administration regimens providing equivalent daily dosages: once daily (QD, 30 μg/kg every 24 hours), twice daily (BID, 15 μg/kg every 12 hours), or three times a day (TID, 10 μg/kg every 8 hours). Frequent administration of ABL showed higher BMD with enhancement of trabecular and cortical bone mass and structures than that of TPTD, consistent with the clinical results seen with once daily administration. ABL increased bone formation marker levels more than TPTD with more frequent regimens, while bone resorption marker levels were not different between ABL and TPTD in all regimens. Analysis of bone histomorphometry and gene expression also suggested that ABL increased bone formation more than TPTD, while the effect on bone resorption

was almost comparable between ABL and TPTD. The bone anabolic windows calculated from bone turnover markers indicated that ABL enhanced the anabolic windows more than TPTD, leading to a robust increase in BMD. The mechanism by which ABL showed a better balance of bone turnover was suggested to be partly due to the enhanced remodeling-based bone formation involved in Ephb4. Taken together, the author’s findings would help elucidate the mechanism by which ABL shows excellent BMD gain and reduction of fractures in patients with osteoporosis.

Introduction

Abaloparatide (ABL) is a novel 34-amino acid peptide analog of parathyroid hormone-related protein (PTHrP) that has recently been approved by Food and Drug Administration (FDA) for the treatment of severe osteoporosis. Clinical trials in postmenopausal women with osteoporosis showed that daily subcutaneous injection of ABL significantly increased bone mineral density (BMD) at the lumbar spine, hip, and femoral neck and reduced the risk of vertebral and non-vertebral fractures.[1,2]

Like teriparatide [TPTD, human parathyroid hormone (PTH) 1-34], ABL also stimulates the PTH/PTHrP receptor (also known as PTH1R) and exerts a bone anabolic effect when administered intermittently.[3-6] However, their effects seem to differ in some respects. Although ABL showed a greater increase in BMD than TPTD, its response to both markers of bone formation and resorption were weaker.[2] These results raise the possibility that ABL stimulates the bone formation marker with a minimum increase in the bone resorption marker, resulting in a greater net bone anabolic window than with TPTD. Still, the effects of ABL on the bone anabolic window and bone mass remain to be elucidated.

The author has previously examined the effects of subcutaneous ABL and TPTD administration on BMD and bone turnover markers in rodents.[7] Unlike the clinical

results, the effects of ABL and TPTD on BMD and bone turnover seemed similar with once daily administration in rodents. Although the reason for the difference between human and animal studies was unclear, species differences in bone turnover should be carefully considered, because bone turnover was reported to be 2 to 3 times higher in rodents than in humans.[8] Therefore, in the present study, the effects of ABL and TPTD administration to mice were compared with several administration regimens ranging from once daily to 3 times a day to understand the mechanism by which ABL shows high efficacy in humans.

Materials and Methods

Peptides

Abaloparatide ([Glu^{22,25}, Leu^{23,28,31}, Aib^{29}, Lys^{26,30}] human PTHrP(1-34)-NH_2) was synthesized by IPSEN (Paris, France). Teriparatide was purchased from BACHEM (Bubendorf, Switzerland). Both ABL and TPTD were dissolved in saline containing 0.1% heat-inactivated mice serum for administration.

Experimental design

All experimental procedures were approved by the Animal Care and Use Committee of Teijin Institute for Bio-Medical Research. Male C57BL6/J mice were purchased from

Charles River Laboratories Japan (Kanagawa, Japan). For all analyses except gene expression, 6-week-old male mice received subcutaneous administration of ABL or TPTD for 28 days (Fig. 1A). The daily dosage was constant at 30 μg/kg/day in all administration groups, and the administration frequency was once daily (QD, 30 μg/kg dose every 24 hours), twice daily (BID, 15 μg/kg dose every 12 hours) or three times a day (TID, 10 μg/kg dose every 8 hours). Mice who received neither ABL nor TPTD were assigned to an intact group. Day 0 indicates the day when administration was started. On day 26, mice were anesthetized with subcutaneous administration of an anesthetic mixture (medetomidine, midazolam and butorphanol). BMD was measured at the lumbar spine (L3 and L4) and the right femur using a PIXImus2 (GE Healthcare, Chicago, IL). Mice were injected subcutaneously with 10 mg/kg calcein 3 and 6 days before sacrifice for analysis of bone mineralization. Blood and urine samples were collected before sacrifice. Prior to collection, mice were fasted for a maximum of 18 hours until euthanasia and kept in metabolic cages to collect pooled urine. On day 28, eight (TID groups), twelve (BID groups) or twenty-four (QD groups) hours after the final administration, mice were anesthetized with subcutaneous administration of an anesthetic mixture, followed by collecting blood from the heart. After euthanasia, mice were perfused with 4% paraformaldehyde diluted in 0.1 mol/L cacodylate buffer

through the left cardiac ventricle. Femora and tibiae were immediately removed, the right femur and tibia were then immersed in the same fixative, the left femur was immersed in 70% ethanol, and the left tibia was immersed in half-strength Karnovsky's fixative for bone histomorphometry, micro-CT, and electron microscopy.

For gene expression analysis, six-week-old male C57BL6/J mice received TID administration of 10 μg/kg ABL or TPTD for 9 days (Fig. 7A). Two, four, or eight hours after the final administration, mice were euthanized, followed by collection of the femora. The femora were kept at -80°C until RNA extraction.

Bone turnover markers and biochemical parameters

Serum procollagen type 1 N-terminal propeptide (P1NP) concentration was measured with a Rat/Mouse PINP EIA kit (Immunodiagnostic Systems, Boldon, UK). Serum C-terminal telopeptide of type I collagen (CTx) concentration was measured with a RatLap ELISA kit (Immunodiagnostic Systems) Urine deoxypyridinoline (DPD) concentration was measured with an Osteolinks DPD kit (Quidel, San Diego, CA). Calcium (Ca) and inorganic phosphate (IP) concentrations in serum and urine and creatinine (Cr) concentration in urine were measured with a 7180 Autoanalyzer (Hitachi High-Technologies Corporation, Tokyo, Japan).

Bone turnover markers are used to calculate bone anabolic windows to assess the net

bone anabolic effect of drugs.[4,9] To calculate anabolic windows, bone turnover marker data were first normalized by dividing marker values of individual animals in the ABL and TPTD administration groups by the average value of the intact group. Normalized values for the resorption markers (urine DPD/Cr and serum CTx) were then subtracted from normalized values for the formation marker (P1NP). Relationships between these anabolic windows versus femur BMD (% change from intact group) were examined by linear regression analysis.

Histochemistry and bone histomorphometry

For alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) staining, the right femur and tibia were decalcified with 10% EDTA-2Na and embedded in paraffin. Dewaxed paraffin sections were examined for ALP staining as reported elsewhere.[10,11] In brief, after inhibition of endogenous peroxidases, dewaxed paraffin sections were pretreated with 1% bovine serum albumin (BSA) (Serologicals Proteins Inc. Kankakee, IL) in PBS (1% BSA-PBS) for 30 minutes. Sections were then incubated for 2–3 hours at room temperature with rabbit polyclonal antisera against ALP,[12] followed by incubation with horseradish (HRP)-conjugated anti-rabbit IgG (DakoCytomation, Glostrup, Denmark). TRAP activity was detected as previously described.[13] In short, slides were rinsed with PBS and incubated in a mixture of

naphthol AS-BI phosphate (Sigma-Aldrich, St. Louis, MO), fast red violet LB salt (Sigma-Aldrich), and L-(+)-tartaric acid in 0.1 mmol/L sodium acetate buffer (pH 5.0) for 15 minutes at 37°C.

For analysis of bone mineralization, the left femur was stained with Villanueva bone stain and embedded in methyl-methacrylate resin without decalcification. The resin was sectioned or polished for fluorescence microscopy.

A 375 µm x 2000 µm (for intact and QD administration groups) or a 750 µm x 2000 µm (for BID and TID administration groups) region of interest (ROI) located 750 µm below the growth plate of the femoral metaphysis was evaluated for assessment of ALP^+ area/tissue area, osteoclast number/bone surface (N.Oc/BS), mineral apposition rate (MAR), mineralized surface/bone surface (MS/BS) and bone formation rate/bone surface (BFR/BS). ALP^+ areas were measured with BZ-H3M software (Keyence, Osaka, Japan). For the evaluation of osteoclast numbers, $TRAP^+$ osteoclasts were counted on bone surfaces. MAR, MS/BS and BFR/BS were measured with Histometry RT digitizer software (System Supply, Nagano, Japan). Each parameter is expressed according to the recommendations of the ASBMR Histomorphometry Nomenclature Committee.[14] All parameters except ALP^+ area/total area were measured by an investigator blinded to the experimental groups.

Micro-CT analysis

Micro-CT images of the left femur were obtained with RmCT (Rigaku, Tokyo, Japan). Reconstructed 3D images were analyzed using TRI/3D-BON software (Ratoc System Engineering, Tokyo, Japan). For trabecular bone structural parameters, cross-sectional slices (total thickness, 3.8 mm) from 0.2 mm below the growth plate of the femoral metaphysis were analyzed. Cross-sectional slices at the femoral diaphysis (total thickness, 1 mm) were analyzed for cortical bone parameters.

Electron microscopic analysis

Transmission electron microscopic (TEM) observation was described previously.[15] In short, the left tibia was immersed in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde solution for 24 h at 4 °C. Decalcified specimens were then post-fixed with osmium tetroxide and embedded in epoxy resin (Epon 812, Taab, Berkshire, UK). Ultra-thin sections were cut with an ultramicrotome (Sorvall MT-5000; Ivan Sorvall, Inc., Norwalk, CT), and stained with uranyl acetate and lead citrate. These specimens were observed under TEM (Hitachi H-7100, Hitachi Co., Tokyo, Japan) at 80 kV.

Gene expression analysis

Total RNA was extracted from femora using an RNeasy Plus Universal Kit (Qiagen, Valencia, CA). RNA was then reverse-transcribed into cDNA using a SuperScrip IV

VIL Master Mix (Thermo Fisher Scientific, Waltham, MA). Quantitative RT-PCR was performed on a StepOnePlus Real Time PCR System (Thermo Fisher Scientific) with a PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). Data are expressed as fold changes after normalization to *gapdh* gene. The primers used in this study are shown in Supplemental Table 1.

Data analysis

All numerical data are expressed as means ± s.e.m. Student's *t*-test or two-way analysis of variance (ANOVA) was used for two-group comparisons. One-way ANOVA followed by Dunnett's test was used for multiple comparisons. For regression analysis, Pearson's correlation coefficient was calculated. Significance was inferred from P values < 0.05. All data were analyzed using GraphPad Prism version 8.2.1 (Graph-Pad Software, La Jolla, CA).

Results

Bone densitometry and microarchitecture

A schematic diagram of the experimental design is shown in Fig. 1A. ABL and TPTD were administered to mice with several administration regimens for 28 days. Consistent with previous studies,[7,16] both ABL and TPTD significantly increased BMD at the

femur and lumbar spine, and their effects were dependent on administration frequency. (Fig. 1B, C). There was no significant difference in BMD between the two peptides when they were administered once daily. However, ABL showed a significant increase in BMD compared with TPTD when the administration regimen was BID or TID (Fig. 1B, C).

The microarchitecture of trabecular and cortical bone was further investigated by micro-CT. Both ABL and TPTD significantly enhanced trabecular bone structural parameters, with increases in trabecular bone volume/total volume (BV/TV), trabecular thickness (Tb.Th), and trabecular number (Tb.N) and a decrease in trabecular separation (Tb.Sp) (Fig. 2A, B). Compared with TPTD, ABL showed a significantly more potent effect on these parameters. ABL and TPTD also significantly increased cortical bone volume (Ct.V) and width (Ct.Wi), and these effects of ABL were significantly greater than of TPTD when the administration regimen was twice daily or more (Fig. 2C, D). These results indicated that frequent administration of ABL increased BMD greater than TPTD with enhancement of trabecular and cortical bone mass and structures, which was consistent with the human results seen with once daily administration.

Bone turnover markers and anabolic window

The bone formation marker serum P1NP was elevated by ABL and TPTD

administration (Fig. 3A). ABL increased serum P1NP significantly more than TPTD with the BID and TID regimens. Both peptides also increased the bone resorption marker urine DPD/Cr (Fig. 3B). However, unlike serum P1NP, no significant difference was found between ABL and TPTD regardless of the administration frequency. A similar trend was observed in another bone resorption marker, serum CTx. ABL and TPTD significantly increased serum CTx, and their effects were not significantly different in all regimens (Fig. 3C).

Bone turnover markers were also used to calculate bone anabolic windows to assess the net bone anabolic effects of drugs by subtracting normalized values of a bone resorption marker from normalized values of a bone formation marker.[4,9] Normalized P1NP was dramatically increased by ABL and TPTD as the frequency of administration increased (Fig. 3D, E). In contrast, the increase in normalized DPD/Cr was modest even in the TID regimen groups. As a result, the anabolic window for the P1NP *vs*. DPD/Cr response was enhanced depending on the administration frequency (Fig. 3F). Of note, ABL showed a significantly greater anabolic window than TPTD. Similar results were obtained in the analysis of the anabolic window for P1NP *vs*. CTx (Fig. 3G-I). Change in normalized CTx was modest with both ABL and TPTD administration (Fig. 3G, H), and the anabolic window for the P1NP *vs*. CTx response was significantly greater with

ABL administration than with TPTD administration (Fig. 3I). Linear regression analysis of anabolic window *vs*. femur BMD (% change from intact group) indicated significant positive correlations with r values of 0.692 for anabolic window [P1NP - DPD/Cr] vs. femur BMD, and 0.686 for anabolic window [P1NP – CTx] vs. femur BMD (Table 1). These results indicated that the enhanced bone anabolic windows with the increased bone formation marker with ABL led to a greater BMD increase than with TPTD.

Biochemical parameters

Serum and urine biochemical parameters showed that neither ABL nor TPTD significantly altered the concentrations of calcium and inorganic phosphate, suggesting little systemic effects on calcium and phosphate with all regimens (Supplemental Table. 2).

Histochemistry and bone histomorphometry

ALP and TRAP are hallmarks for osteoblastic cells and osteoclasts, which are responsible for bone formation and resorption, respectively. To compare the effects of ABL and TPTD in bone tissue, histochemical detection for ALP and TRAP was assessed in femoral metaphyses. ALP immunoreactivity became broader and more intense with ABL and TPTD depending on the administration frequency. (Fig. 4A). ABL showed significantly higher ALP^+ area/total area than TPTD in all regimens (Fig.

4C). The number of $TRAP^+$ osteoclast was also increased by ABL and TPTD with more frequent administrations (Fig. 4B). No significant difference was observed in N.Oc/BS between ABL and TPTD with the QD and BID regimens, while a significantly weaker effect was found with ABL administration than with TPTD administration with the TID regimen (Fig. 4D).

To investigate the effect on bone mineralization, fluorescence-labeled bone specimens of mice who received ABL or TPTD with the TID regimen were analyzed (Fig. 5A, C). Both ABL and TPTD significantly increased MAR, MS/BS, and BFR/BS at the femoral metaphysis and diaphysis (Fig. 5B, D). ABL showed a significantly greater increase in BFR/BS at both sites than TPTD, indicating a potent effect of ABL on bone mineralization in both trabecular and cortical bone. Taken together, histomorphometrical analysis suggested that ABL promoted bone formation more than TPTD, while its effect on bone resorption was equivalent to or less than that of TPTD.

Electron microscopic observation

TEM observation showed mature osteoblasts on the bone surface in specimens of ABL- or TPTD-administered mice with the TID regimen. (Fig. 6A, B). There were also preosteoblasts adjacent to osteoblasts (Fig. 6A, B). Interestingly, preosteoblasts developed more cytoplasmic processes with ABL administration than with TPTD

administration (Fig. 6C, D).

Gene expression

PTH acts on osteoblasts and osteocytes and regulates several genes related to bone formation.[17] It also stimulates osteoclast bone resorption by regulating osteoclastogenesis genes such as receptor activator of nuclear factor-kappa B (RANKL) and its decoy receptor osteoprotegerin (OPG).[17] To compare the effect of ABL and TPTD on gene expression, mice received ABL or TPTD with the TID regimen for 9 days, and femora were collected 2, 4 and 8 hours after the final administration (Fig. 7A). ABL and TPTD altered bone formation-related genes with increases in *alpl*, *col1a1*, and *ephb4* and a decrease in *sost*, a gene encoding sclerostin that inhibits osteoblast differentiation (Fig. 7B-E).[18] ABL showed significantly more potent effects on *alpl*, *col1a1* and *ephb4* than TPTD (Fig. 7B-D). No significant difference was found in *sost* expression between ABL and TPTD (Fig. 7E). ABL and TPTD also altered gene expression related to osteoclastogenesis and bone resorption including *rankl*, *opg*, *rankl/opg* and *trap* (Fig. 7F-I). Although ABL increased *rankl* expression significantly more than TPTD, the effects on other osteoclastogenesis and bone resorption genes were not different between ABL and TPTD (Fig. 7F-I). These results suggested that ABL promoted bone formation-related genes more than TPTD, while its effect on the

bone resorption-related gene seemed comparable, consistent with the results of bone turnover markers and histomorphometry.

Discussion

In humans, ABL showed a greater BMD increase with weaker responses to both bone formation and resorption markers than TPTD.[1,2] These profiles of ABL make it difficult to understand the relationship between the bone anabolic window (increase in formation versus resorption) and bone mass. Thus, the present study compared the effects of ABL and TPTD on BMD and bone turnover in mice. Given that the rate of bone turnover is higher in rodents than in humans,[8] the comparison was made with several administration regimens ranging from QD to TID. With QD administration, the effect on BMD was not different between ABL and TPTD. Of note, TID administration of ABL showed greater BMD increases at both the femur and lumbar spine than TID administration of TPTD, consistent with the clinical results. These results suggest that TID administration to mice is a suitable regimen to assess the clinical efficacy of ABL and TPTD by once daily administration. Micro-CT analysis showed that frequent administration of ABL showed greater increases in cortical bone as well as trabecular bone than TPTD. These findings provide insight into the clinical efficacy of ABL which

shows a robust BMD increase at the hip and femoral neck, which are abundant in cortical bone.

Bone turnover markers showed that ABL promote bone formation more potently than TPTD, while the effect on bone resorption was almost comparable. Analysis of histomorphometry and gene expression also showed a similar trend, which further supports these findings. The anabolic window is often used to assess the net bone anabolic effect of drugs.[4,9] The author's data clearly indicate that ABL enhanced the anabolic window more than TPTD, leading to the robust increase in BMD. The responses of ABL to bone turnover, including both bone formation and resorption, seemed greater in mice than in humans. Although the reason is unclear, the author's findings suggest that the greater anabolic window of ABL brought by a higher ratio of bone formation versus resorption provides a robust BMD increase and reduction of fracture in patients with osteoporosis.

The mechanism by which ABL shows a more favorable bone turnover state is not fully understood. PTH1R agonists are reported to promote bone formation in both modeling-based and remodeling-based manners.[19] Modeling-based bone formation, in which sclerostin is involved, occurs independent of bone resorption.[20] The author showed that ABL and TPTD inhibited the *sost* gene to a similar extent, suggesting that

modeling-based bone formation was not different between the two peptides. Remodeling-based bone formation, which is coupled with bone resorption, is regulated by osteoclast-derived factor, namely coupling factor.[21] Ephrinb2-ephb4 is one of the coupling factor-related signals, where osteoclast ephrinb2 directly contacts its receptor ephb4 on preosteoblasts, leading to osteoblast differentiation and bone formation with reduced osteoclast differentiation.[22] The expression of Ephb4 is reported to be regulated by PTH.[23] The author found that ABL also promotes the *ephb4* gene, and its effect was greater than that of TPTD. In addition, electron microscopic observation showed more cytoplasmic processes of preosteoblasts with ABL administration. Cytoplasmic processes are important for cell migration or cell-cell direct interactions. Previous studies have shown that cytoplasmic processes were regulated by PTH1R signaling in preosteoblast and osteocyte.[24,25] Considering these results, one proposed hypothesis is that morphological change and enhancement of the *ephb4* gene by ABL administration might help the direct interaction of preosteoblasts with osteoclasts and evoke a subsequent bone formation signal via Ephb4, leading to a more favorable state of bone turnover than with TPTD administration. The involvement in other coupling factors and the mechanism by which ABL shows more cytoplasmic processes remains to be elucidated. Further research will be necessary to better understand the mechanism

of action of ABL on bone turnover.

Finally, this study has some limitations. The anabolic window was calculated from the bone turnover markers at the end of treatment, not at multiple time points from the start of treatment. Chronological data on the bone turnover markers would provide a more accurate anabolic window of drugs. Another limitation is that bone strength was not evaluated in this study. Bone strength is an important factor based on its relevance to fracture risk reduction. Further study will be required to better understand the effect of ABL.

In conclusion, the present study showed that the administration of ABL to mice increased BMD more than TPTD, as in humans when the frequency of administration was adjusted by the rate of bone turnover. The author also found that ABL enhanced the anabolic window more than TPTD, leading to bone gain including trabecular and cortical bone. The mechanism by which ABL shows a favorable balance of bone turnover may be partly due to enhanced remodeling-based bone formation. These findings would help elucidate the mechanism by which ABL shows excellent BMD gain and reduction of fractures in patients with osteoporosis.

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Figure Legends

Fig. 1. ABL shows a higher BMD increase than TPTD with more frequent administration regimens. (A) A schematic diagram of the experimental design. Mice received subcutaneous ABL or TPTD injection for 28 days in QD (every 24 hours), BID (every 12 hours) and TID (every 8 hours) regimens. The daily dosage is constant at 30 μg/kg/day in all regimens. (B) BMD at the femur and lumbar spine on day 26. n = 6 (Intact) or 13 (QD, BID and TID groups). **P*<0.05, ***P*<0.01, Student's *t*-test, #*P*<0.05, ###*P*<0.001, vs. Intact, Dunnett's test.

Fig. 2. ABL enhances trabecular and cortical bone mass and structures more than TPTD. (A and C) Micro-CT images of the distal femur and diaphysis. (B and D) Structural parameters of trabecular and cortical bone. n = 6 (Intact) or 13 (QD, BID and TID groups). ***P*<0.01, ****P*<0.001, Student's *t*-test, ##*P*<0.01, ###*P*<0.001, vs. Intact, Dunnett's test.

Fig. 3. ABL shows enhanced anabolic windows with increased bone formation marker levels. (A) Serum P1NP, (B) urine DPD/Cr, and (C) serum CTx were measured. (D and E) P1NP and DPD/Cr data were normalized by dividing values of individual

animals by the average value of the intact group. (F) The anabolic window was calculated by subtracting normalized P1NP values from normalized DPD/Cr values (G-I) A similar analysis was performed in D-F. n = 6 (Intact) or 12-13 (QD, BID, and TID groups). *P<0.05, **P<0.01, Student's t-test, #P<0.05, ###P<0.001, vs. Intact, Dunnett's test, NS, not significant.

Fig. 4. ABL increases the ALP$^+$ area more than TPTD, while the effect on osteoclast number is equivalent or less. Histological images of the distal femur with (A) ALP staining and (B) TRAP staining. (C) The percentage of ALP$^+$ area was measured from ALP-stained specimens (D) TRAP$^+$ osteoclast number per bone surface was counted from TRAP-stained specimens. n = 6 (Intact) or 13 (QD, BID and TID groups). *P<0.05, ***P<0.001, Student's t-test, #P<0.05, ###P<0.001, vs. Intact, Dunnett's test, NS, not significant.

Fig. 5. ABL shows a greater effect on bone mineralization than TPTD. Histological images fluorescence-labeled bone at the (A) femoral metaphysis and (C) femoral diaphysis. (B and D) Mineralization parameters of trabecular bone at the metaphysis and periosteal cortical bone at the diaphysis were measured. n = 6 (Intact) or 13 (ABL

and TPTD). *P<0.05, **P<0.01, ***P<0.001, Student's t-test, ###P<0.001, vs. Intact, Dunnett's test.

Fig. 6. ABL shows more cytoplasmic processes of preosteoblasts. TEM images of the tibial metaphysis. Asterisks and arrows indicate preosteoblasts (pre-ob) and cytoplasmic processes, respectively. Scale bar = 10 μm (A and B), 5 μm (C and D). ob, osteoblast.

Fig. 7. ABL and TPTD regulate the expression of genes related to bone formation and resorption. (A) A schematic diagram of the experimental design. Mice received TID administration of 10 μg/kg ABL or TPTD for 9 days. Femora were collected 2, 4 and 8 hours after the final administration. (B-I) The expression of genes related to bone formation and resorption was measured by quantitative RT-PCR. Data are expressed as fold changes after normalization to *gapdh* gene. n = 10, *P<0.05, *P<0.05, ***P<0.001, two-way ANOVA, #P<0.05, ##P<0.01, ###P<0.001, vs. Vehicle (at corresponding time point), Dunnett's test.

Table 1. Linear regression analysis of anabolic window *vs*. femur BMD.

Parameter	Pearson's r value	*P* value
[P1NP - DPD/Cr] vs. BMD	0.692	< 0.0001
[P1NP - CTx] vs. BMD	0.686	< 0.0001

Figure. 1

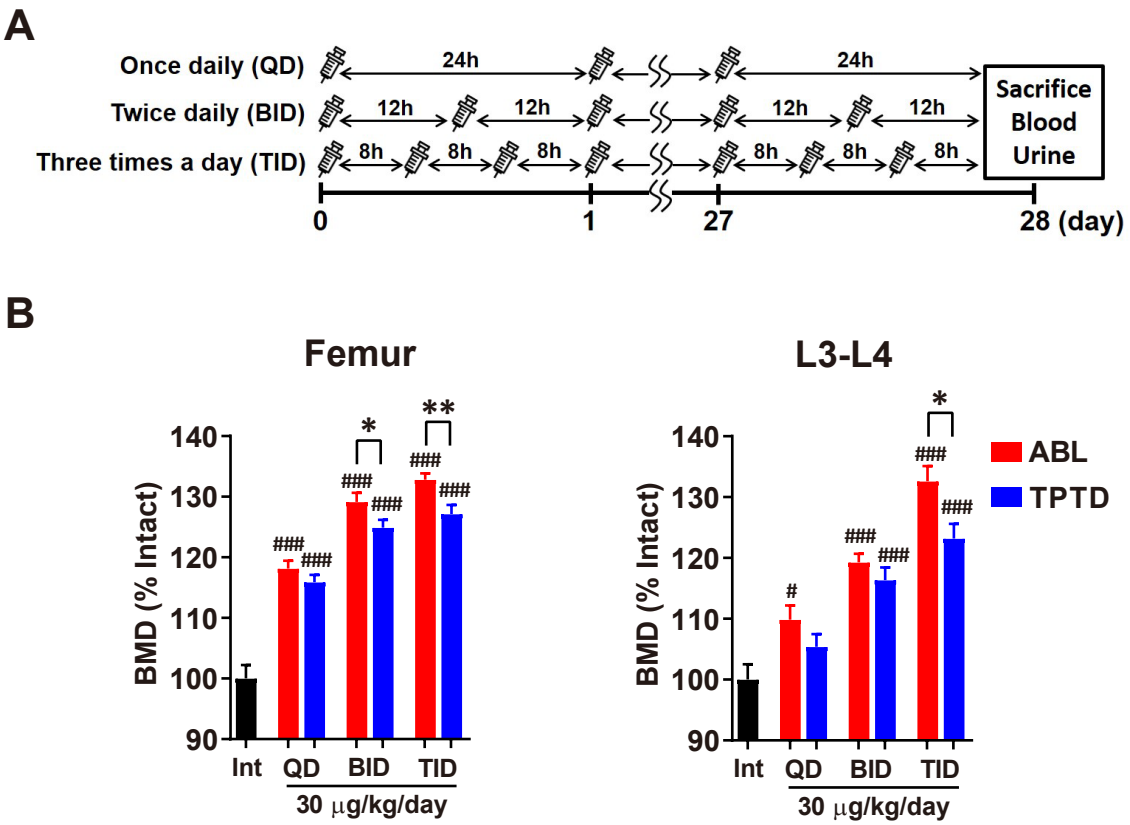

A
Once daily (QD)
Twice daily (BID)
Three times a day (TID)
24h
12h
8h
Sacrifice
Blood
Urine
0
1
27
28 (day)
B
Femur
L3-L4
BMD (% Intact)
ABL
TPTD
Int
QD
BID
TID
30 μg/kg/day

Figure. 2

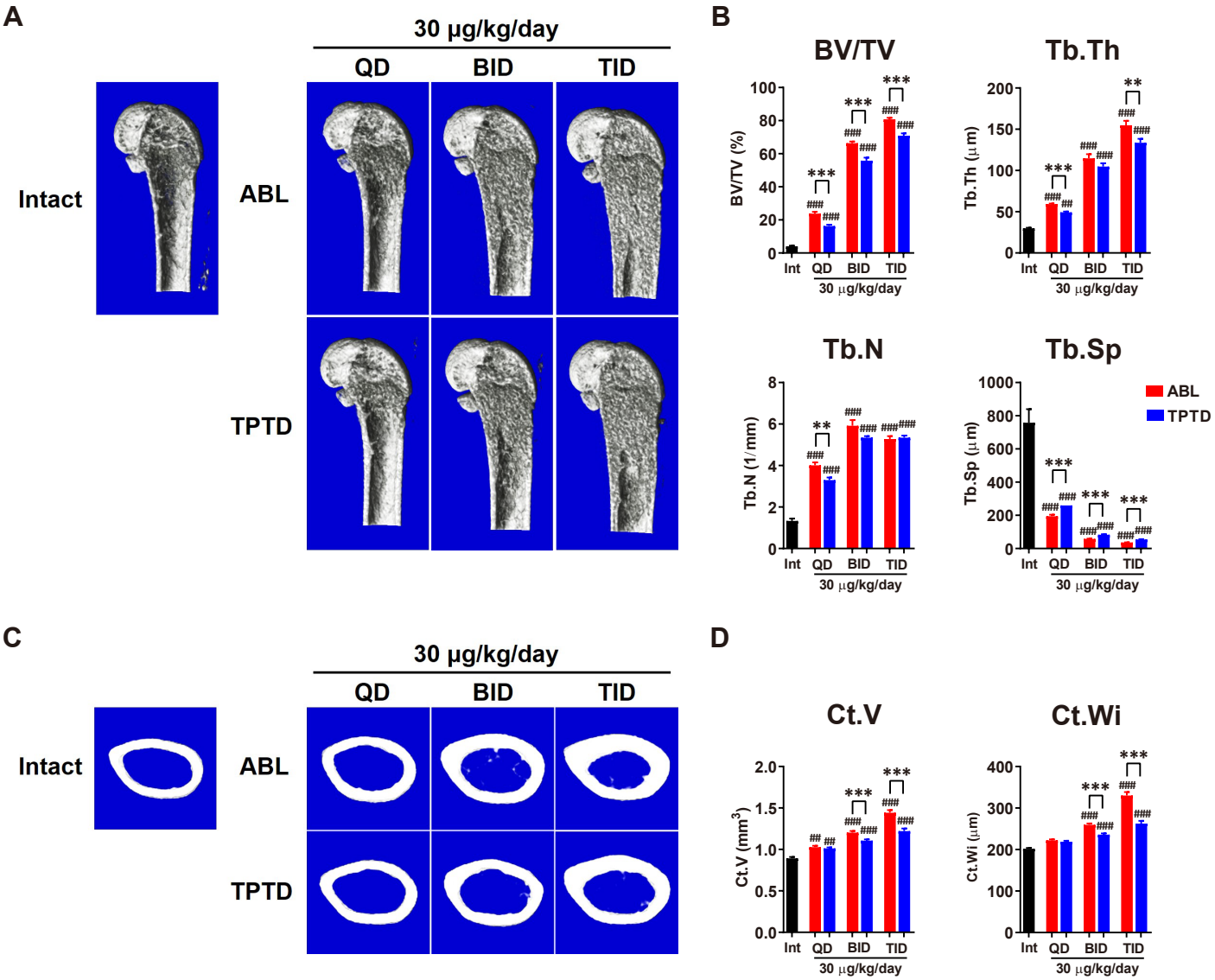

A
30 μg/kg/day
QD
BID
TID
Intact
ABL
TPTD
B
BV/TV
BV/TV (%)
Tb.Th
Tb.Th (μm)
Tb.N
Tb.N (1/mm)
Tb.Sp
Tb.Sp (μm)
ABL
TPTD
Int
QD
BID
TID
30 μg/kg/day
C
30 μg/kg/day
QD
BID
TID
Intact
ABL
TPTD
D
Ct.V
Ct.V (mm3)
Ct.Wi
Ct.Wi (μm)
Int
QD
BID
TID
30 μg/kg/day

Figure. 3

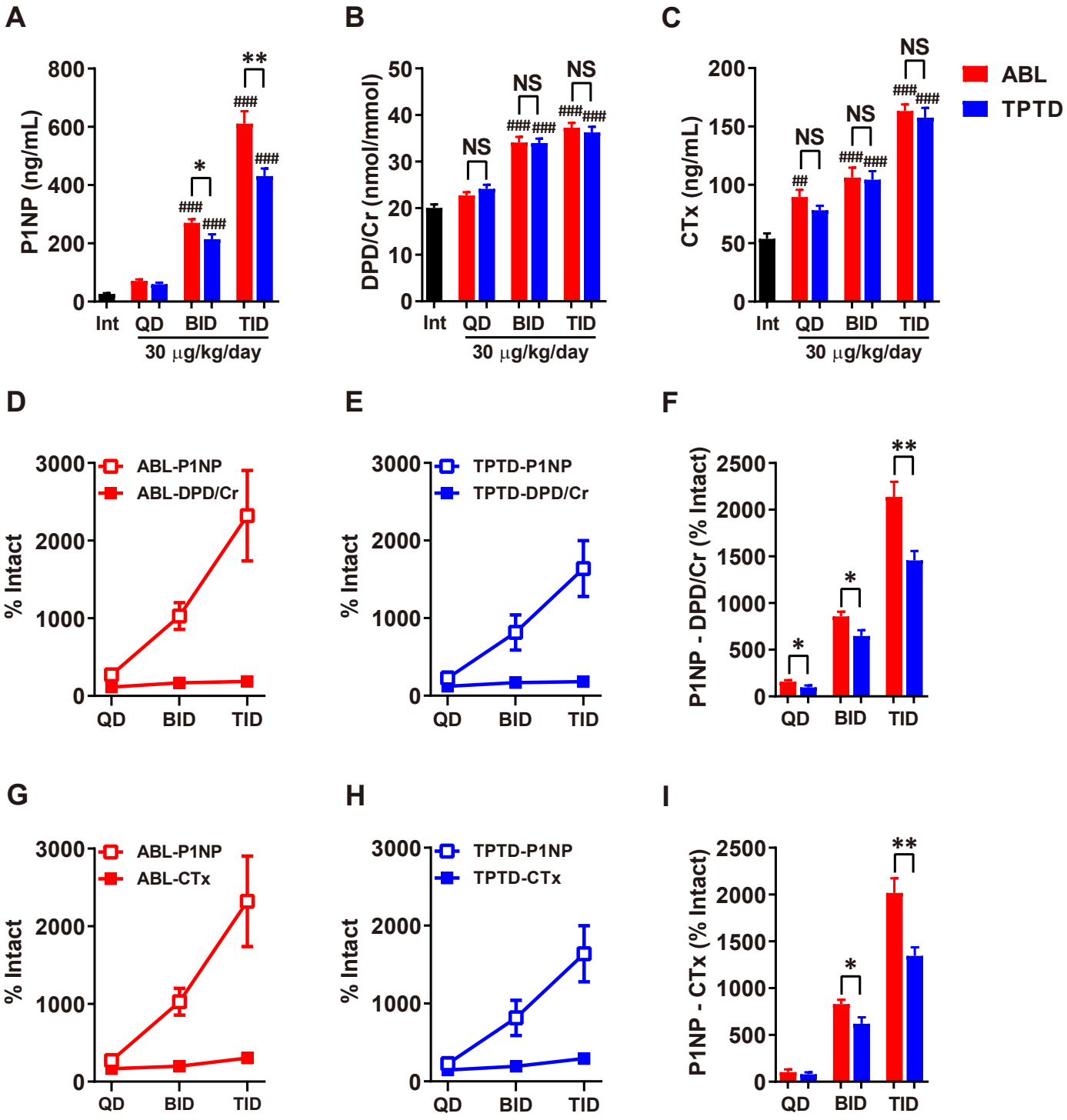

A
P1NP (ng/mL)
800
600
400
200
0
Int
QD
BID
TID
30 μg/kg/day
*
**
###
B
DPD/Cr (nmol/mmol)
50
40
30
20
10
0
NS
###
C
CTx (ng/mL)
200
150
100
50
0
##
ABL
TPTD
D
% Intact
3000
2000
1000
0
ABL-P1NP
ABL-DPD/Cr
E
TPTD-P1NP
TPTD-DPD/Cr
F
P1NP - DPD/Cr (% Intact)
2500
2000
1500
1000
500
G
ABL-CTx
H
TPTD-CTx
I
P1NP - CTx (% Intact)

Figure. 4

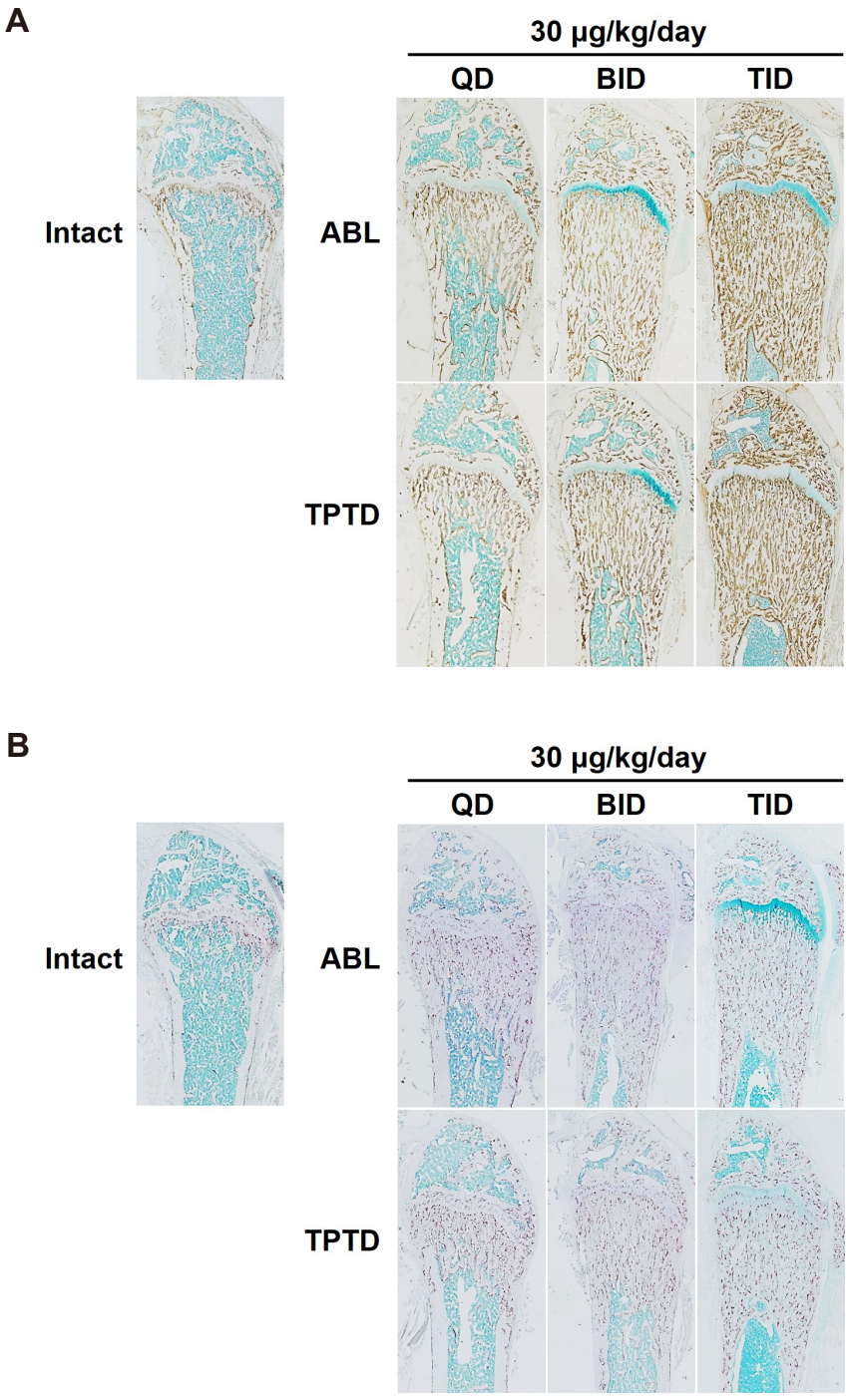

A
30 μg/kg/day
QD
BID
TID
Intact
ABL
TPTD
B
30 μg/kg/day
QD
BID
TID
Intact
ABL
TPTD
C
ALP+ Ar/T.Ar (%)
50
40
30
20
10
0
Int
QD
BID
TID
30 μg/kg/day
*
***
*
###
###
###
###
D
N.Oc/BS (N/mm)
10
8
6
4
2
0
NS
NS
*
ABL
TPTD
###
###
#
#
#
#
Int
QD
BID
TID
30 μg/kg/day

Figure. 5

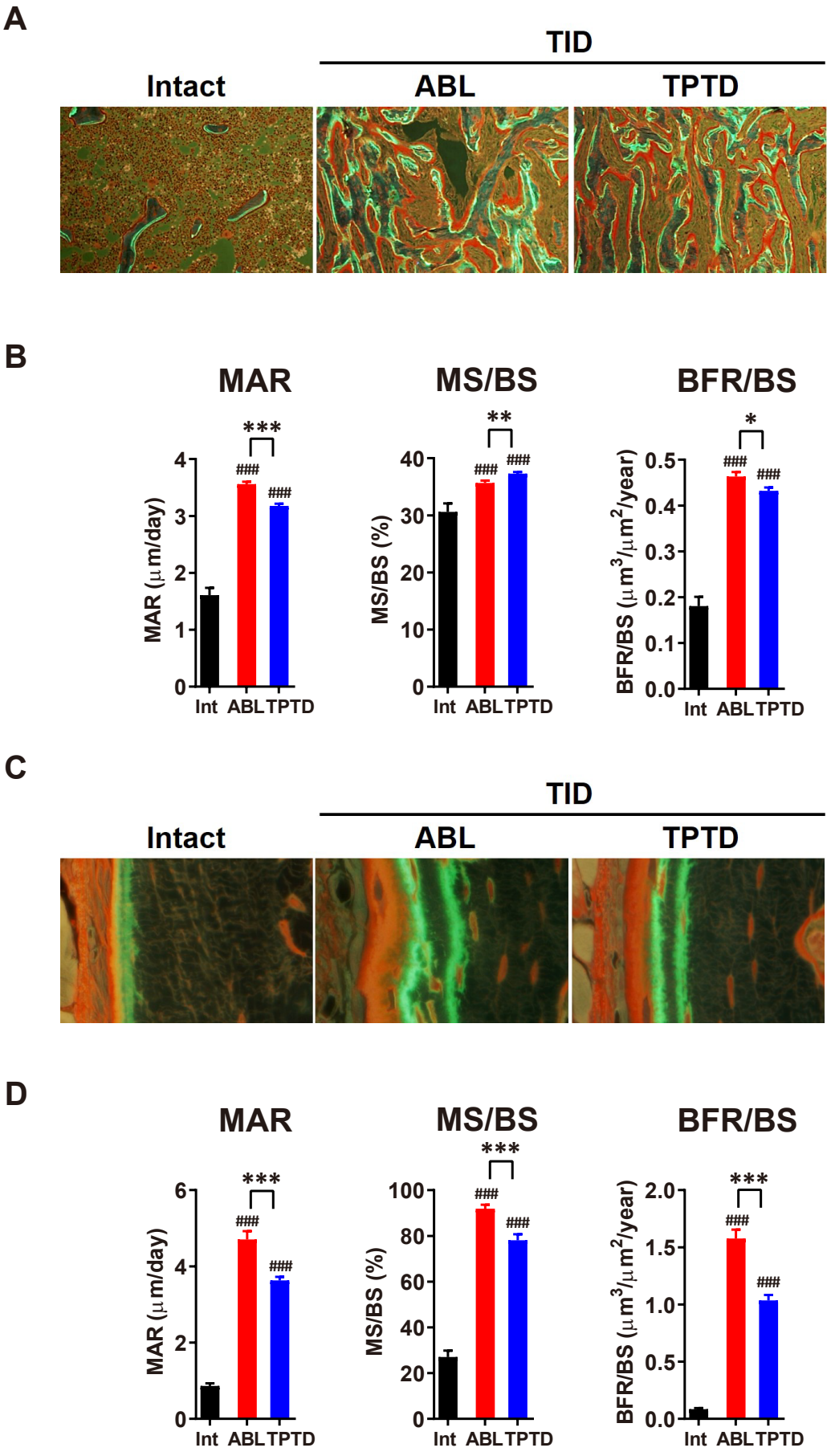

A
TID
Intact
ABL
TPTD
B
MAR
MS/BS
BFR/BS
MAR (μm/day)
MS/BS (%)
BFR/BS (μm³/μm²/year)
Int ABL TPTD
C
TID
Intact
ABL
TPTD
D
MAR
MS/BS
BFR/BS
MAR (μm/day)
MS/BS (%)
BFR/BS (μm³/μm²/year)
Int ABL TPTD

Figure. 6

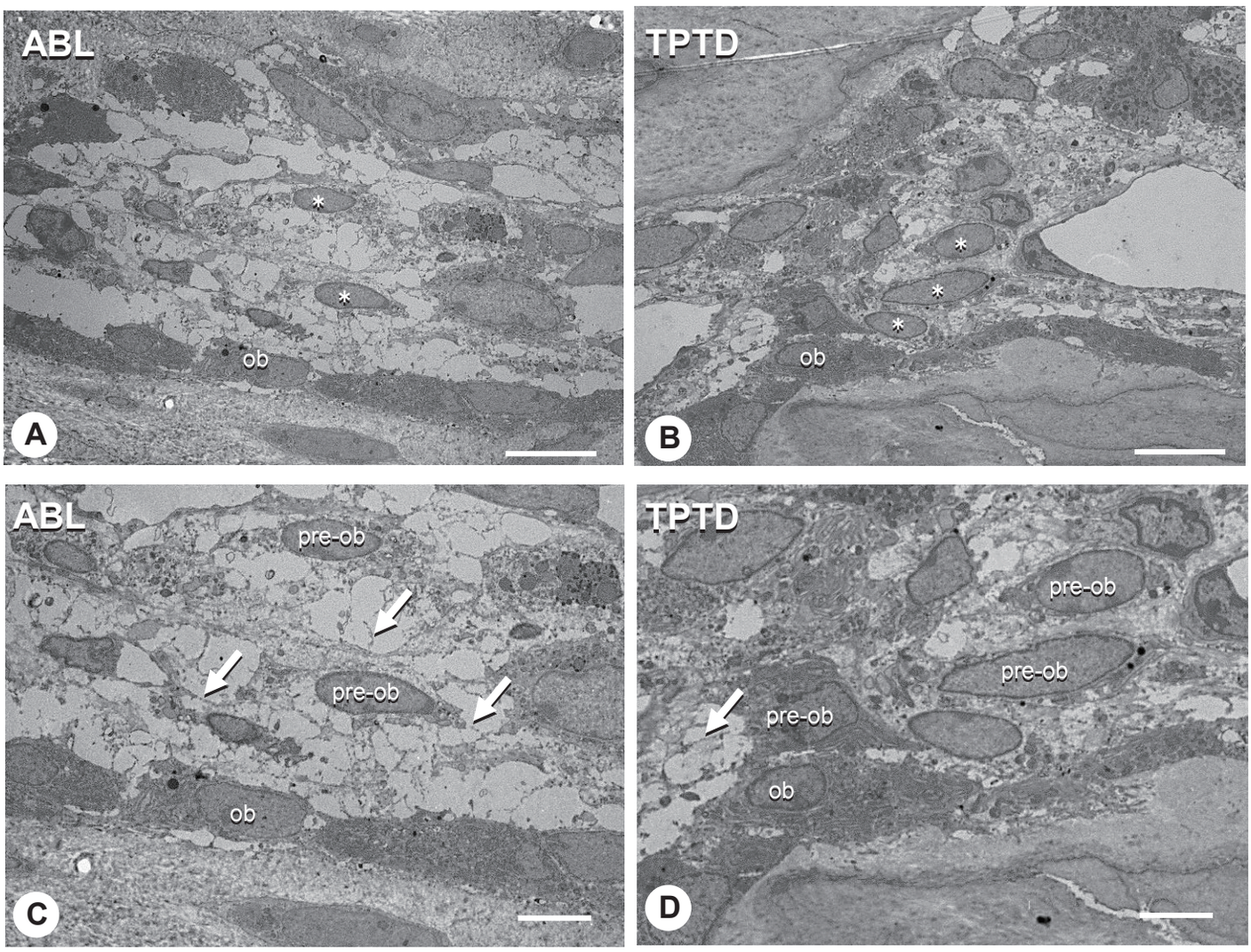
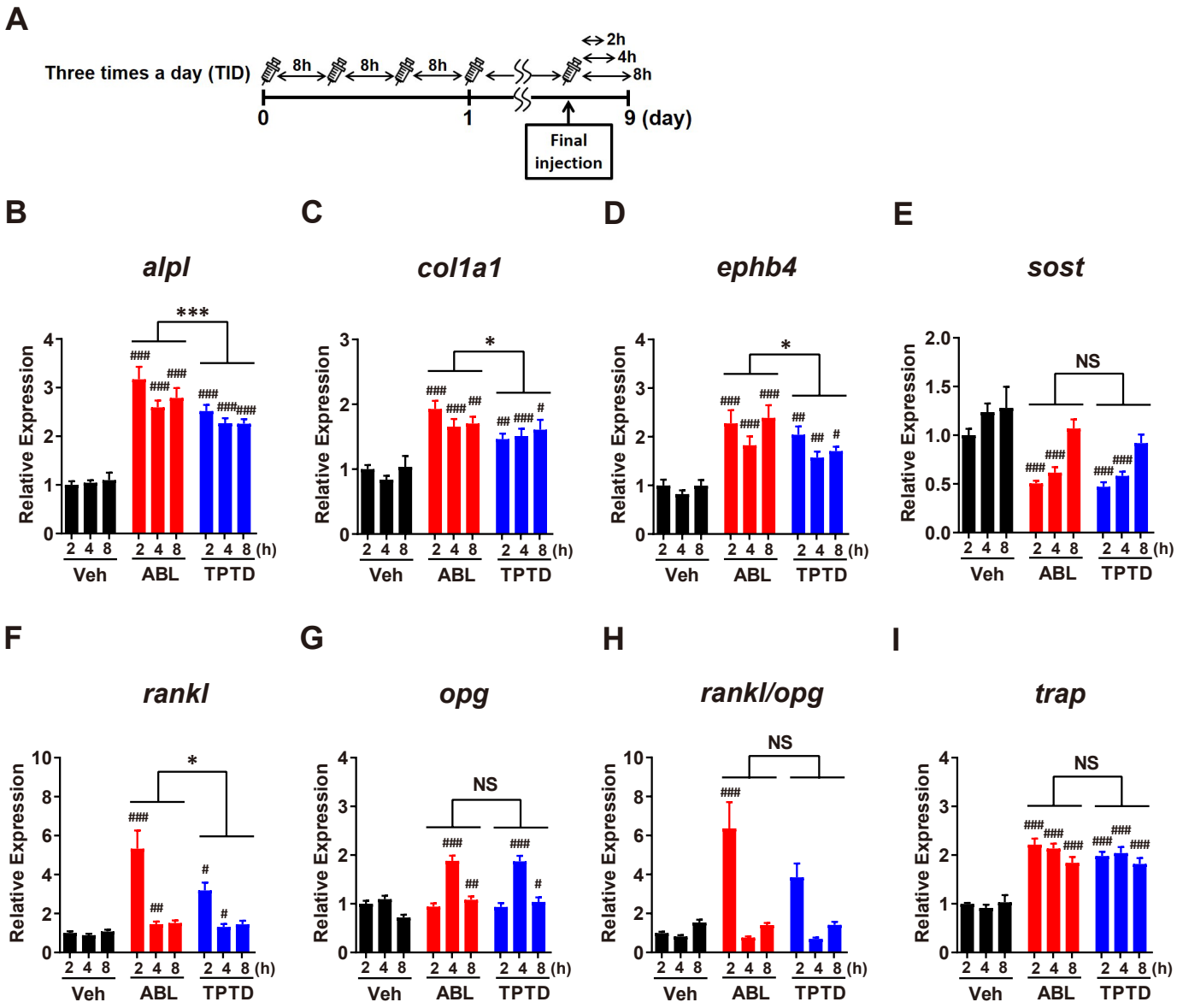

ABL
ob
A
TPTD
ob
B
ABL
pre-ob
pre-ob
ob
C
TPTD
pre-ob
pre-ob
pre-ob
ob
D

Figure. 7


A
Three times a day (TID)
8h
8h
8h
2h
4h
8h
0
1
9 (day)
Final injection
B
alpl
C
col1a1
D
ephb4
E
sost
F
rankl
G
opg
H
rankl/opg
I
trap
Relative Expression
Veh
ABL
TPTD
(h)
NS

Supplemental Data

Supplemental Table 1. Primers used in this study

Gene	Forward Primer	Reverse Primer
alpl	GTTGCCAAGCTGGGAAGAACAC	CCCACCCCGCTATTCCAAAC
col1a1	CCACGTCTCACCATTGGGG	GACTGTCTTGCCCCAAGTTC
ephb4	AGTGGCTTCGAGCCATCAAGA	CTCCTGGCTTAGCTTGGGACTTC
gapdh	GGATGCAGGGATGATGTTC	TGCACCACCAACTGCTTAG
opg	ACAGTTTGCCTGGGACCAAA	TCACAGAGGTCAATGTCTTGGA
rankl	CCTGAGGCCCAGCCATTT	CTTGGCCCAGCCTCGAT
sost	ATCTGCCTACTTGTGCACGC	CGGACATCTTTGGCGTC
trap	GCTGTCCTGGCTCAAAAAGC	CACACCGTTCTCGTCCTGAA

Supplemental Table 2. Serum and urine biochemical parameters

parameter	Intact	QD ABL	QD TPTD	BID ABL	BID TPTD	TID ABL	TID TPTD
Serum Ca (mg/dL)	9.78 ± 0.20	10.16 ± 0.13	9.49 ± 0.11	9.74 ± 0.11	9.79 ± 0.12	9.52 ± 0.23	9.53 ± 0.20
Serum IP (mg/dL)	11.38 ± 1.39	13.33 ± 0.56	12.98 ± 0.61	12.49 ± 0.54	13.11 ± 0.48	12.92 ± 0.76	11.84 ± 0.35
Urine Ca/Cr (mg/mg)	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	0.16 ± 0.01	0.20 ± 0.03
Urine IP/Cr (mg/mg)	9.26 ± 0.35	9.28 ± 0.27	8.50 ± 0.67	9.43 ± 0.37	9.29 ± 0.51	7.64 ± 0.61	8.46 ± 0.40

Data are expressed as means ± s.e.m. n = 6 (Intact) or 13 (ABL and TPTD).