



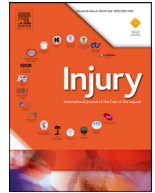
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## Soft tissue injury in the limbs increased regional bone turnover

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### ABSTRACT

**Background:** Pathological conditions after skeletal tissue injury such as trauma and surgical intervention are often accompanied with regional osteoporotic changes, which are recognized to be mainly caused by limb immobility after injury. However, the mechanisms for the progression of regional osteoporotic changes related to the injury remains unknown. Previous studies reported that the pathophysiological conditions related to tissue injury include the acidic micro-environment formation and increased ATP levels. In addition, we previously demonstrated that those changes in the micro-environment induced a high bone turnover state through the activation of TRPV1, ASICs and P2X expressed in bone cells. We, therefore, hypothesized that tissue injury could enhance a high bone turnover state due to those pathophysiological changes in soft tissue in the injured limb. The aim of this study was to examine whether soft tissue injury associated with cutaneous incisions in a limb affects regional bone turnover.

**Methods:** Eight-week-old male C57BL/6 J mice underwent soft tissue injury associated with cutaneous incisions in the right femoral skin. During the 14 days after the incision, changes in the expression of osteoblast and osteoclast differentiation regulators and ATP were evaluated in comparison with those in uninjured mice. The pain-like behaviors and the expression of those differentiation regulators with and without treatment with bisphosphonate and Cox2 inhibitor were assessed in the injured limb.

**Results:** Consistent with the wound healing process, the expression levels of Osterix, osteocalcin and RANKL in the femur of the incised limb were significantly increased up to 7 days, and then decreased to the same level as those in the control limbs by 14 days after the incisions. The levels of TRAP 5b and ATP were initially significantly increased, and then decreased to the same level as before injury by day 14. Bisphosphonate significantly improved the pain-like behaviors in the injured limb associated with the inhibition of osteoblast and osteoclast differentiation regulators.

**Conclusion:** We believe that the pathophysiological changes in soft tissue resulting from cutaneous incisions could be related to the induction of osteoblast and osteoclast differentiation regulators.

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### Introduction

Pathological conditions after skeletal tissue injury such as trauma and surgical intervention, are often accompanied with regional osteoporotic changes. Such pathological changes are recognized to be mainly caused by limb immobility after injury [1]. In rare cases, the osteoporotic changes progress markedly with an accompanying development of refractory skeletal pain, such as complex regional pain syndrome (CRPS) [2]. However, the mechanisms for the progression of regional osteoporotic changes related to trauma and skeletal invasions remains unknown.

Previous studies demonstrated that bone cells expressed acid-sensing receptors, including transient receptor potential channel vanilloid subfamily member 1 (TRPV1) and acid sensing ion channels (ASICs), as well as ATP receptors, including P2Xs [3,4]. These receptors are activated in response to noxious chemical stimulation such as the formation of an acidic micro-environment and increase in ATP level [5-8]. In addition, bone turnover was enhanced through chemical stimulation-induced activation of the receptors on bone cells [9]. On the other hand, it is known that noxious molecules and inflammatory cytokines are released in the periphery of injured soft tissues resulting from trauma or surgical intervention [10-12]. Those noxious molecules in the pathological soft tissues might directly affect bone turnover through the activation of receptors expressed on bone cells in the injured limbs.

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We, therefore, hypothesized that soft tissue injury induces the formation of a pathological environment around the bone in injured tissues, and such changes could enhance regional bone turnover in the injured limbs. The aim of this study was to examine whether soft tissue injury in a limb affects the regional bone turnover in the affected limb using a mouse model.

## Materials and methods

### Animals

The present experiments were approved by the University Animal Care Committee (Reference number, 20-071) and were undertaken in accordance with the ethical guidelines of the National Institute of Health. Every effort was made to minimize animal suffering and the number of animals used. Experiments were conducted on 8-week-old male C57BL/6 J mice weighing 20–25 g (Japan SLC, Hamamatsu, Japan). The mice were housed in a temperature-controlled room ( $21\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ ) with a 12 h light/dark cycle and were given free access to food and water. The animals were humanely sacrificed with an intraperitoneal injection of pentobarbital sodium (0.5 mg/kg) after completion of the experiments. We used a total of 112 mice, consisting of 78 cutaneous-incised (injured) and 34 non-injured (control) mice. Fifty-two mice were used for the assessment of pain-like behaviors (32 injured and 20 control mice), of which 32 mice were also used for the excision of the femoral bone (16 injured and 16 control mice). Thirty-six mice were used for measurement of tartrate-resistant acid-phosphatase (TRAP) 5b and ATP levels (28 injured and 8 control mice). Twenty-four mice were used for the excision of the femoral bone in the drug-treatment experiments (18 injured and 6 control mice). Assessment of pain-like behavior was performed before, at 1, 3, 7 and 14 days after the femoral cutaneous incision. The right femur and blood samples were collected before, at 1, 3, 7 and 14 after the incision for evaluation of the levels of osteoblast and osteoclast differentiation regulators. A drug was administered to the mice subcutaneously once a day for 14 days after the incision.

### Cutaneous incision model

A single full-thickness cutaneous incision of 15 mm in length was made with a scalpel in the right femoral skin as a soft tissue injury model. The wounds were neither dressed nor sutured as described in our previous study [13]. The right hind limbs of non-injured mice were used as a control. We monitored mouse activity for 10 min using a video camera to assess their level of movement at 1, 7 and 14 days after cutaneous incision. We confirmed that the stress on the mice in our experiments could be regarded as minimal based on the fact that there were no significant changes in body weight.

### Assessment of pain-like behavior

Behavioral tests were performed, as previously described [4,14,15], prior to cutaneous incision and at 1, 3, 7 and 14 days after injury. In brief, to assess the mechanical withdrawal response (von Frey test), the mice were placed in plastic chambers above a wire mesh floor that allowed full access to the hind-paw. A 1.34 g von Frey filament (Semmes-Weinstein Monofilaments, North Coast Medical Inc., San Jose, CA, USA) was used to produce mechanical tactile stimuli, which were applied to the middle area of the plantar surface of the hind-paws. Each hind-paw was probed consecutively by 10 stimulations. This was repeated at least 5 times at intervals of at least 10 min, and the final value was obtained by averaging the 5 measurements. Mechanical sensitivity was evaluated as the rate of withdrawal responses. Visible lifting of the

stimulated hind limb was considered to be a withdrawal response [15,16]. Thermal nociceptive testing (paw-flick test) was conducted using an analgesimeter (Plantar test 7370, Ugo Basile, Italy). The mice were placed in plastic chambers ( $6 \times 4 \times 4\text{ cm}$ ) and left unrestrained. Radiant heat was applied to the plantar surface of the hind-paw until it was actively withdrawn by the animal. The thermal withdrawal response was measured as the latency of the hind-paw withdrawal. Paw withdrawal latency (PWL) was considered to be an index of the thermal nociceptive threshold, and a decrease in this measurement indicated thermal hyperalgesia. The light beam intensity was adjusted so that the basal PWL was 7–10 s. The cut-off time was set at 15 s to avoid tissue damage [4,14,15]. We used these tests as surrogate methods for measuring skeletal pain in the limbs as described in previous studies [4,14,15]. The observer was blinded to the treatment regimen received by the mice.

### Evaluation of the expression levels of osteoblast and osteoclast differentiation regulators in the femur

For RNA isolation and reverse transcription-polymerase chain reaction, the right femur from the mice with a right femoral cutaneous incision, and the right femur from the mice without any incision (control) were excised after humane sacrifice under general anesthesia and individually homogenized in 1 ml TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) 10 times at 3000 rpm/30 s using a bead crusher (TITEC, Tokyo, Japan). The homogenates were centrifuged at  $15,000 \times g$  for 15 min at  $4\text{ }^{\circ}\text{C}$ , and the supernatants were then extracted. Total RNA was individually isolated from the femurs from each mouse, and then reverse transcribed (RT) into cDNA by polymerase chain reaction (PCR) using an RNA PCR kit (PrimeScript™ RT-PCR Kit; Takara Bio Inc., Tokyo, Japan) according to the manufacturer's protocol. The primers used were the same as those in our previous study (Table 1) [17]. Reactions were carried out by PCR as follows; 35 cycles at  $95\text{ }^{\circ}\text{C}$  for 40 s,  $55\text{ }^{\circ}\text{C}$  for 40 s and  $72\text{ }^{\circ}\text{C}$  for 40 s for GAPDH; 35 cycles at  $95\text{ }^{\circ}\text{C}$  for 30 s,  $64\text{ }^{\circ}\text{C}$  for 30 s and  $72\text{ }^{\circ}\text{C}$  for 30 s for RANKL; and 30 cycles at  $95\text{ }^{\circ}\text{C}$  for 30 s,  $64\text{ }^{\circ}\text{C}$  for 30 s and  $72\text{ }^{\circ}\text{C}$  for 30 s for Runx2, Osterix and osteocalcin. The values of the target gene bands were normalized to the level of GAPDH gene expression in the same sample for semi-quantitative measurement. Values in graphs are the means  $\pm$  SD obtained from 4 independent experiments.

### Measurements of femoral bone and serum tartrate-resistant acid-phosphatase (TRAP) 5b levels

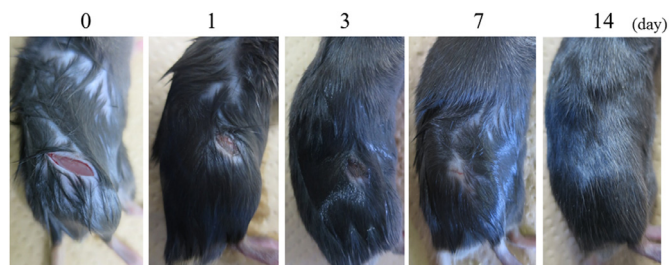
Bone and blood samples were collected before and after the incision. The femoral bone samples were taken and cleaned of all soft tissues, which were used for the assay of TRAP5b as a bone resorption marker. The femur was homogenized in 300 ml physiological saline at  $40,000\text{ rpm}/30\text{ s} \times 2$  times using a bead crusher. The homogenates were centrifuged at  $15,000 \times g$  for 10 min at  $4\text{ }^{\circ}\text{C}$  and the supernatants were then extracted. The blood samples were centrifuged at  $15,000 \times g$  for 15 min at  $4\text{ }^{\circ}\text{C}$  and the supernatants were extracted for preparation of the serum sample. TRAP5b values were measured using a mouse TRAP5b enzyme-linked immune sorbent assay kit (Immunodiagnostic Systems, London, UK) in accordance with the manufacturer's recommendations.

### Measurement of serum ATP level

Blood samples were collected from the mice before and after the incision. For serum preparation,  $100\mu\text{l}$  of the samples were homogenized with ATP assay buffer for 20 s, centrifuged at  $10,000 \times g$  for 5 min at  $4\text{ }^{\circ}\text{C}$ , and the supernatants were then extracted. The serum ATP concentration was determined us-

**Table 1**  
Primer sequence.

	Forward primer	Reverse primer
Runx2	GCTTGATGACTCTAAACCTA	AAAAAGGGCCAGTTCTGAA
Osterix	AGGCACAAAGAAGCCATAC	AATGAGTGAGGGAAGGGT
Osteocalcin	CTCACTCTGCTGGCCCTG	CCGTAGATGCGTTTGTAGGC
Rankl	ATCAGAAGACAGCACTCACT	ATCTAGGACATCCATGCTAATGTTT
Gapdh	TGAAGTCGGTGTGAACGAATT	GCTTCTCCATGGTGGTGAAGA



**Fig. 1.** Wound healing processes after the right femoral cutaneous incision. The incised wounds were spindle-shaped with exposure of the underlying muscle fascia (day 0), and covered by a dehydrated wound crust or scab at day 1. The scab was generally gone at day 7, and the wounds fully healed by day 14 after the incision.

ing an AMERIC-ATP kit (Applied Medical Enzyme Research Inc., Tokushima, Japan) according to the manufacturer's protocol.

#### Administration of drugs

Alendronate (0.02 mg/kg body weight diluted in physiological saline) (ALN; Merck & Co. Inc., NJ), a potent anti-resorptive agent, was administered to the mice subcutaneously [14] once a day for 14 days after the femoral cutaneous incision. Carprofen (5 mg/kg body weight diluted in physiological saline) (carprofen; Sigma-Aldrich Japan, Tokyo, Japan), a Cox2 inhibitor agent, was administered to the mice subcutaneously [84] once a day for the same period as ALN administration.

#### Statistical analysis

All data are presented as means  $\pm$  standard deviation. To determine differences between groups, measurements were repeated at least three times for each sample, and the individual mean value was used for the statistical analysis. The statistical significance between 2 groups was determined using a Student's *t*-test, and that among 3 groups was determined using ANOVA followed by Tukey's post-hoc test. Differences with *p* values of  $< 0.05$  were considered to be statistically significant.

## Results

### The wound healing process after femoral cutaneous incision

Immediately after the incision in the right femur, the wounds were spindle-shaped with well-separated incision edges and exposure of the underlying muscle fascia. The wounds were covered by a dehydrated wound crust or scab at day 1 after incision. The scab was progressively lost and generally absent at day 7, revealing a thin residual skin defect. By day 14 after the incision, the wounds were fully healed, based on the macroscopic closure of the incision interface and restoration of epithelial coverage (Fig. 1).

### Pain-like behaviors in mice with femoral cutaneous incisions

During the 14 days after the femoral cutaneous incision, pain-like behaviors as assessed by the von Frey test and paw-flick test were significantly increased in comparison with those in the control mice. After 14 days, there were no significant differences in pain-like behaviors between the mice with and without cutaneous incisions (Fig. 2A and B). The level of movement in the mice with femoral cutaneous incisions did not differ significantly from that in the control mice (Fig. 2C).

### Changes in the expression levels of osteoblast and osteoclast differentiation regulators in the femoral bone after femoral cutaneous incisions

We examined whether pathological changes in the soft tissue injured by cutaneous incision affected the expression levels of osteoblast and osteoclast differentiation regulators in the femoral bone. The expression levels of Osterix at day 1 (Fig. 3A, B) and 7 (Fig. 3E, F), osteocalcin at day 7 (Fig. 3E, F), and RANKL at day 1 (Fig. 3A, B), 3 (Fig. 3C, D) and 7 (Fig. 3E, F) were significantly increased in the femoral bone after the incision compared to the values in the femoral bone of the control mice (Fig. 3). At day 14 (Fig. 3G, H) after injury, there were no significant differences in the expression levels of those regulator between the incised and control mice. Throughout the 14 days after the incision, there was no significant increase in Runx2 expression (Fig. 3).

### Tartrate-resistant acid-phosphatase 5b (TRAP5b) levels in the femoral bone marrow and serum

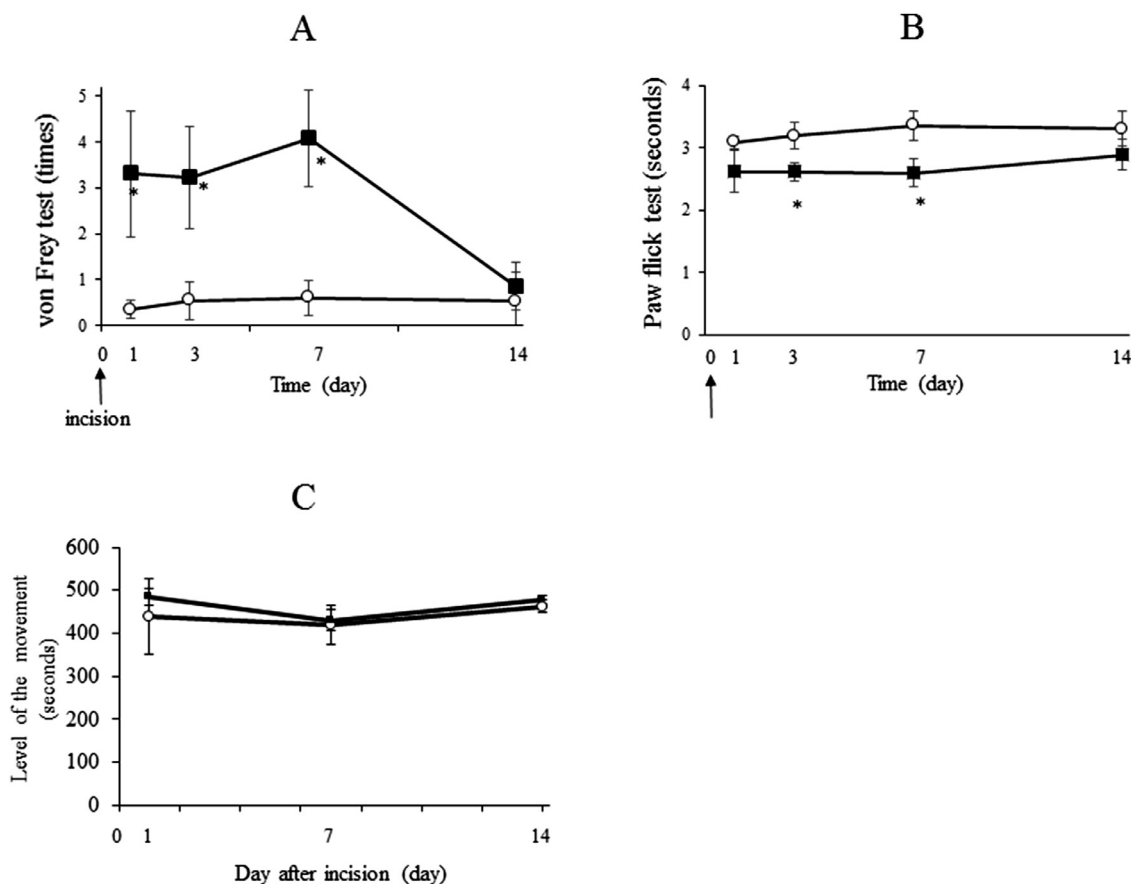
The level of TRAP-5b (U/L) in the femoral bone marrow was significantly increased at day 1, 3 and 7 after the cutaneous incision compared with that before the incision, with the peak observed at day 1 (Fig. 4A). The serum level of TRAP5b was also found to be significantly increased, which was consistent with that observed in the femoral bone (Fig. 4B).

### Changes in serum ATP level after the cutaneous incision in the limb

The serum ATP level was significantly increased at day 1 after the cutaneous incision, and rapidly decreased to the same level as that before the incision by day 3 (Fig. 5).

### Effects of bisphosphonate and Cox2 inhibitor (carprofen) on pain-like behaviors in mice with femoral cutaneous incisions

ALN treatment for 14 days after the incision significantly improved pain-like behaviors as assessed by the von Frey test (Fig. 6A) and paw-flick test (Fig. 6B) at day 7 and 14. These significant improvements were initially recognized from day 7 after the start of ALN treatment, but tended to be partial in comparison with those in the control mice (Fig. 6A, B). We evaluated the anti-inflammatory effects on pain-like behaviors in the injured limb of the incised mice of the administration of a Cox2 inhibitor (carprofen) for 14 days. Carprofen significantly and immediately improved



**Fig. 2.** Pain-like behaviors in mice with femoral cutaneous incisions

At 1, 3 and 7 days after the femoral cutaneous incision (black squares,  $n = 4$ ), the pain-like behavior as assessed by the von Frey test (A) and paw-flick test (B) were significantly induced in comparison with those in the control mice (white circles,  $n = 4$ ). At 14 days, there were no significant differences in those behaviors between the mice with and without cutaneous incisions. The movement time per 10 min in the mice with femoral cutaneous incisions (black squares,  $n = 4$ ) was not significantly different from that in the control mice (white circles,  $n = 4$ ) at day 1, 7 and 14 after the incision (C). Tukey's post-hoc test, \* $p < 0.05$ .

pain-like behaviors from day 1 after the start of treatment (Fig. 6C, D). However, the level of pain-like behavior as assessed by the von Frey test still tended to be higher than that in the control mice (Fig. 6C). In addition, we examined the effects of treatment with both ALN and carprofen on pain-like behaviors (Fig. 6E, F). Simultaneous treatment significantly and completely improved pain-like behaviors as assessed by the von Frey test and paw-flick test from day 1 after the start of treatment in the injured limb to the same level as that in the control mice (Fig. 6E, F).

#### *Effects of bisphosphonate and Cox2 inhibitor (carprofen) on the expression of osteoblast and osteoclast differentiation regulators in femoral bone after cutaneous incisions*

We examined the effects of ALN ( $n = 6$ ) and carprofen ( $n = 6$ ) on the expression of osteoblast and osteoclast differentiation regulators in the femur at day 7 after cutaneous incisions. ALN significantly inhibited the increased expression of the regulators including Runx2, osteocalcin and RANKL in the femur with cutaneous incisions (Fig. 7A and B). On the other hand, the expression levels of these regulators were not significantly changed by treatment with carprofen (Fig. 7A and B).

#### **Discussion**

Osteoporosis is described as a systemic skeletal disease characterized by decreased bone mass and altered microarchitecture in the bone tissue, leading to enhanced bone fragility and risk of

fractures [18]. Recent studies have indicated that the high bone turnover state observed in osteoporosis is induced by noxious molecules, such as proton ( $H^+$ ) and ATP, through activation of those receptors expressed on bone cells [9,17,19] as well as by changes in the hormonal balance and mechanical load on the limbs. In addition, previous studies demonstrated that the levels of those molecules were increased under the high bone turnover conditions resulting from increased  $H^+$  [3,14] and ATP [20,21] production from active osteoclasts and osteoblasts. Furthermore, regional osteoporosis induction was prevented by treatment with antagonists to acid-sensing and ATP receptors due to inhibition of osteoclast and osteoblast function through receptor inactivation. [9,15,17].

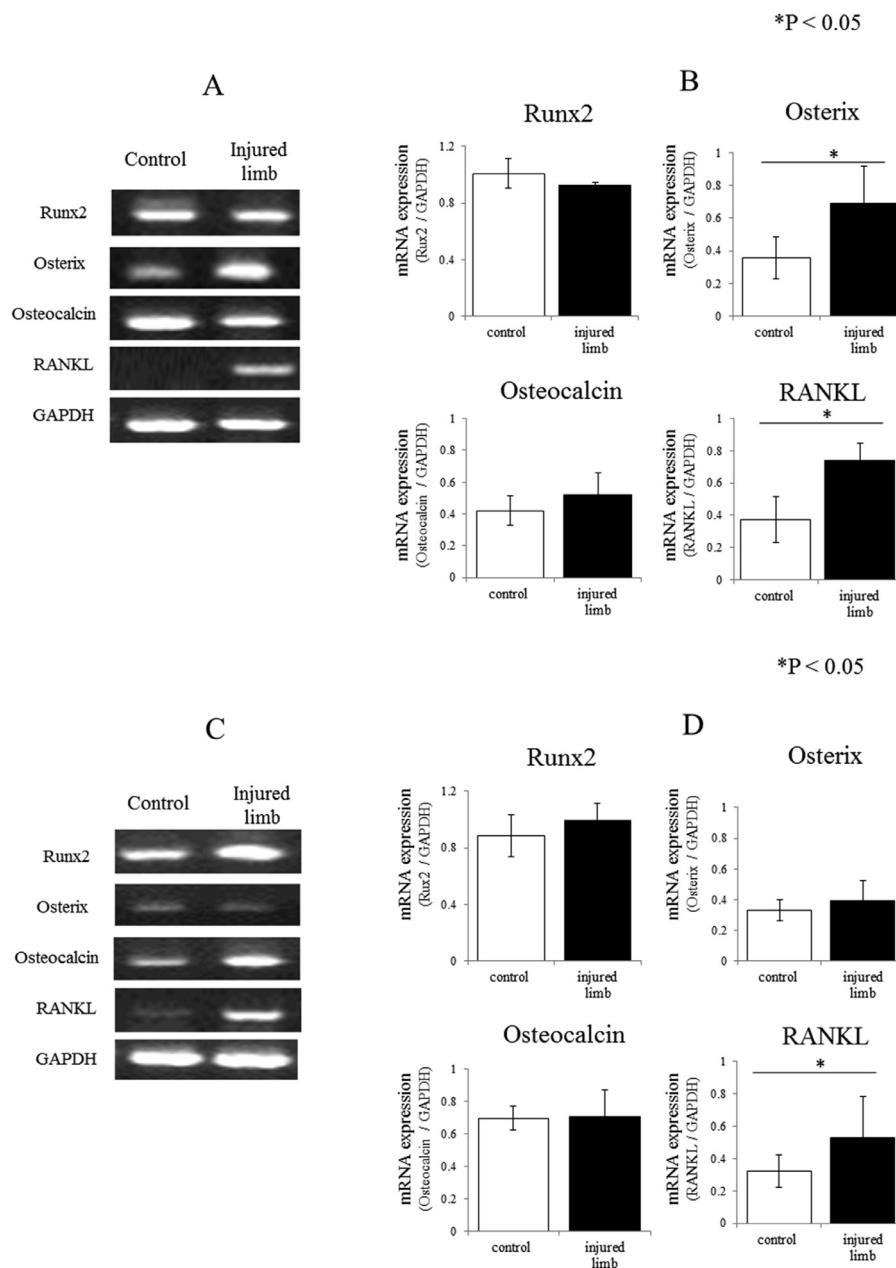
On the basis of those studies [3,9,14,15,17,19-21], we have reported one of the mechanisms for the triggering of regional osteoporosis as follows. Firstly, a pathological micro-environment, such as high levels of  $H^+$  or ATP, is triggered by the induction of osteoclastic bone resorption. These conditions activate osteoblasts and osteoclasts through the stimulation of acid-sensing and ATP receptors including TRPV1, ASICs and  $P2 \times 2/3$  expressed on bone cells [3,4]. Subsequently, these activated bone cells further secrete  $H^+$  and ATP, or inflammatory cytokines, such as IL-1, IL-6 and TNF- $\alpha$ , resulting in the further induction of high bone turnover. The continuation of this pathophysiological cycle is speculated to induce progressive regional bone loss [17]. Regarding this mechanism for the triggering of regional bone loss, we hypothesized that the pathophysiological changes in the injured soft tissues of limbs even without bone injury could possibly be the first trigger to directly

induce regional bone turnover in the injured limb through receptor activation on the bone cells by stimulation of those molecules, as the noxious molecules such as  $H^+$  and ATP are known to be released from the periphery of injured soft tissues resulting from trauma or surgical intervention [10-12].

In this study, the wound resulting from the cutaneous incision was covered by a dehydrated crust or scab at day 1, which was progressively lost and generally absent at day 7, revealing a thin residual skin defect, with the wounds fully healed by day 14 after the incision. Consistent with that wound healing process, pain-like behaviors in the mice and the expressions levels of osteoblast and osteoclast differentiation regulators including Osterix, osteocalcin and RANKL in the femur of the cutaneous-incised limb were significantly increased up to day 7, and then decreased to the same levels as those in the hind limbs of the control mice by day 14

after the incisions. The TRAP5b level, an osteoclast differentiation marker, in the femoral bone marrow and serum was also significantly increased up to day 7 after the incision, with the peak observed at day 1. By day 14, the TRAP5b level had decreased to the same level as that before the incision. The ATP level was significantly increased at day 1 after the injury, and then immediately returned to the same level as that before the injury. These results indicated that the pathophysiological changes resulting from the soft tissue injury associated with cutaneous incisions could be related to the induction of osteoblast and osteoclast differentiation regulators corresponding to a high bone turnover condition.

Regarding the injured model mouse, a decrease in the movement in the mice with femoral cutaneous incisions is thought to be a contributing factor in the changes in the expression levels of osteoblast and osteoclast differentiation regulators. However, we did



**Fig. 3.** Changes in the expression levels of osteoblast and osteoclast differentiation regulators in the femoral bone after the cutaneous incision

The expression levels of Osterix at day 1 (A, B) and 7 (E, F), osteocalcin at day 7 (E, F), and RANKL at day 1 (A, B), 3 (C, D) and 7 (E, F) were significantly increased in the femoral bone after the cutaneous incision (injured limb, black bar,  $n = 6$ /day) compared to those in the limbs of the control mice (control, white bar,  $n = 6$ /day). At day 14 (G, H), there were no significant differences in the expression levels of those regulator. Tukey's post-hoc test,  $*p < 0.05$ .

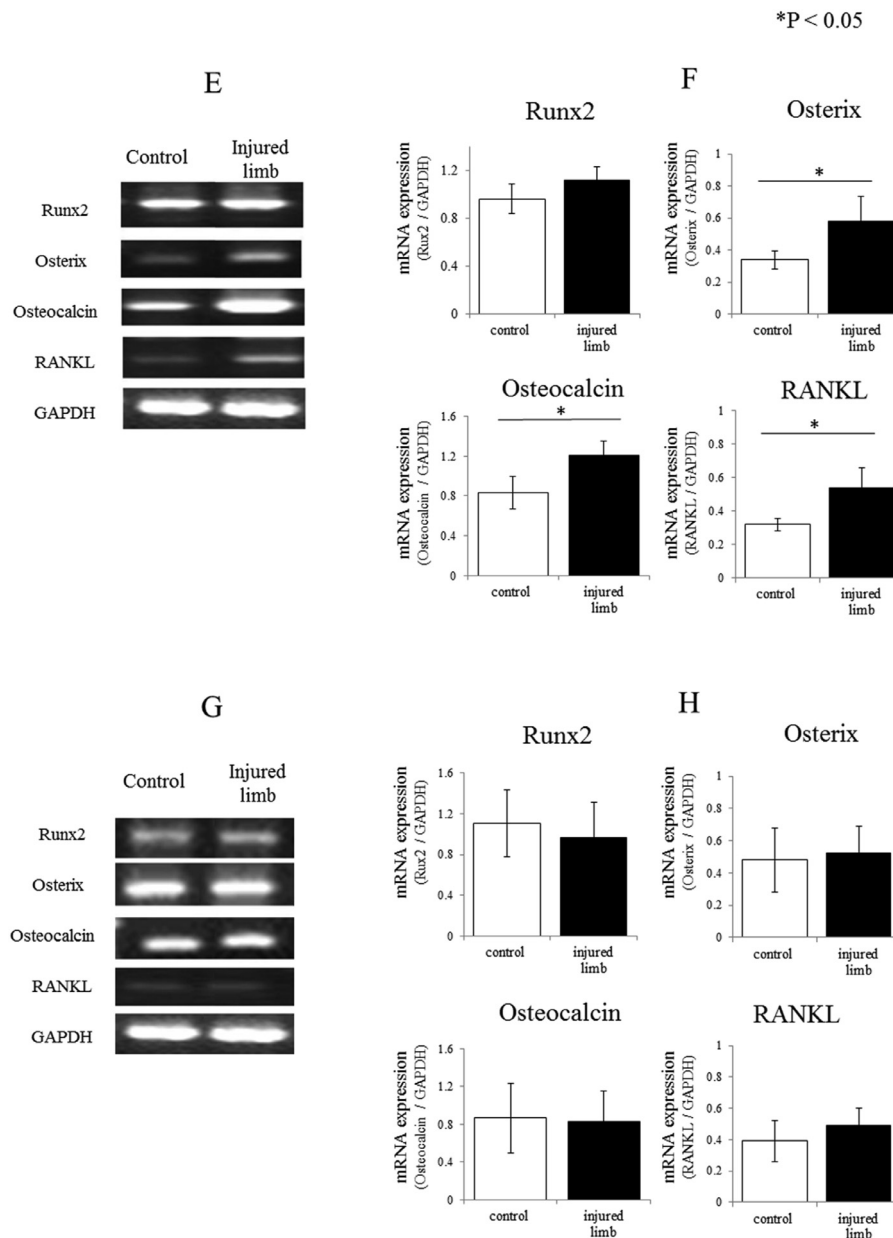
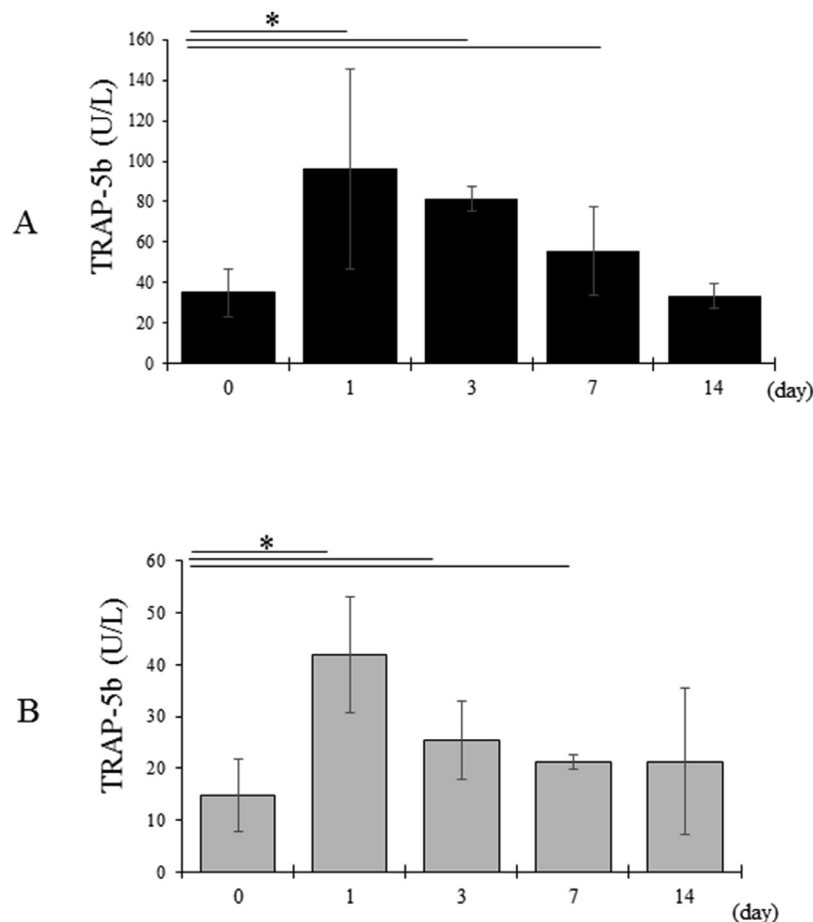


Fig. 3. Continued

not find any significant differences in the level of movement between the mice with a femoral cutaneous incision and the control mice. Thus, we believe that the level of movement did not contribute in a major way to the changes in expression of the regulators examined in this study. Thus, we hypothesize that the pathophysiological changes in the injured soft tissues of limbs could induce regional bone turnover through the activation of receptors such as TRPV1, ASICs and P2 × 2/3 in the bone cells resulting from noxious molecule stimulation. (Fig. 8)

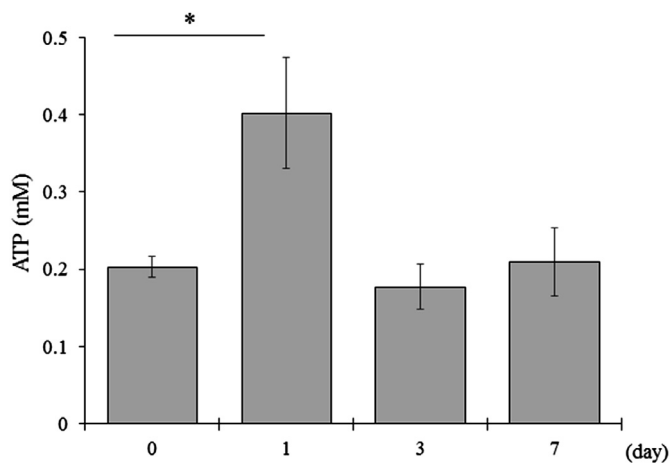
Recent studies demonstrated that bisphosphonate, an anti-osteoporosis agent, significantly improved pain-like behaviors in osteoporosis model mice accompanied with improvement in the increased levels of osteoblast and osteoclast differentiation regulators [4,15,17]. In the present study, we demonstrated that ALN significantly and gradually improved the increased pain-like behaviors resulting from cutaneous incisions from day 7 after the injury, whereas the Cox2 inhibitor significantly and immediately improved them from day 1. Interestingly, simultaneous treatment with ALN

and Cox2 completely and immediately improved pain-like behaviors to the same level as that in the control, while individual treatment with ALN or the Cox2 inhibitor showed partial improvement in pain-like behaviors. Regarding the mechanisms underlying the improved pain-like behaviors, ALN significantly inhibited the expression of osteoblast and osteoclast differentiation regulators increased by the soft tissue injuries; however, the Cox2 inhibitor had no significant effect on those regulators. We, therefore, think that the mechanism underlying the improvement in pain-like behaviors by treatment with ALN could differ from that by treatment with the Cox2 inhibitor. The beneficial effect of ALN might occur through the inhibition of a high bone turnover state, and that of the Cox2 inhibitor might occur through inhibition of inflammatory changes in soft tissue injured by the cutaneous incision. Several studies indicated that bisphosphonate might directly suppress the release of noxious molecules and inflammatory cytokines from the injured tissue [22]. However, most of the administered bisphosphonate is taken up by bone tissue within 24 h and remains in the



**Fig. 4.** Tartrate-resistant acid-phosphatase 5b (TRAP5b) levels in the femoral bone marrow and serum

The TRAP-5b levels in the femoral bone marrow of the injured limb (black bar,  $n = 8/\text{day}$ , A) and serum level of TRAP5b (grey bar,  $n = 8/\text{day}$ , B) were significantly increased at day 1, 3 and 7 after the cutaneous incision compared with that before the incision (day 0), with the peak observed at day 1. Student's  $t$ -test,  $*p < 0.05$  (versus the level before the cutaneous incision).



**Fig. 5.** Changes in serum ATP level after the cutaneous incision in the limb

The serum ATP levels were significantly increased at day 1 after the cutaneous incision, and rapidly decreased to the same level as that before the incision by day 3 ( $n = 4/\text{day}$ ). Student's  $t$ -test,  $*p < 0.05$  (versus the level before the cutaneous incision).

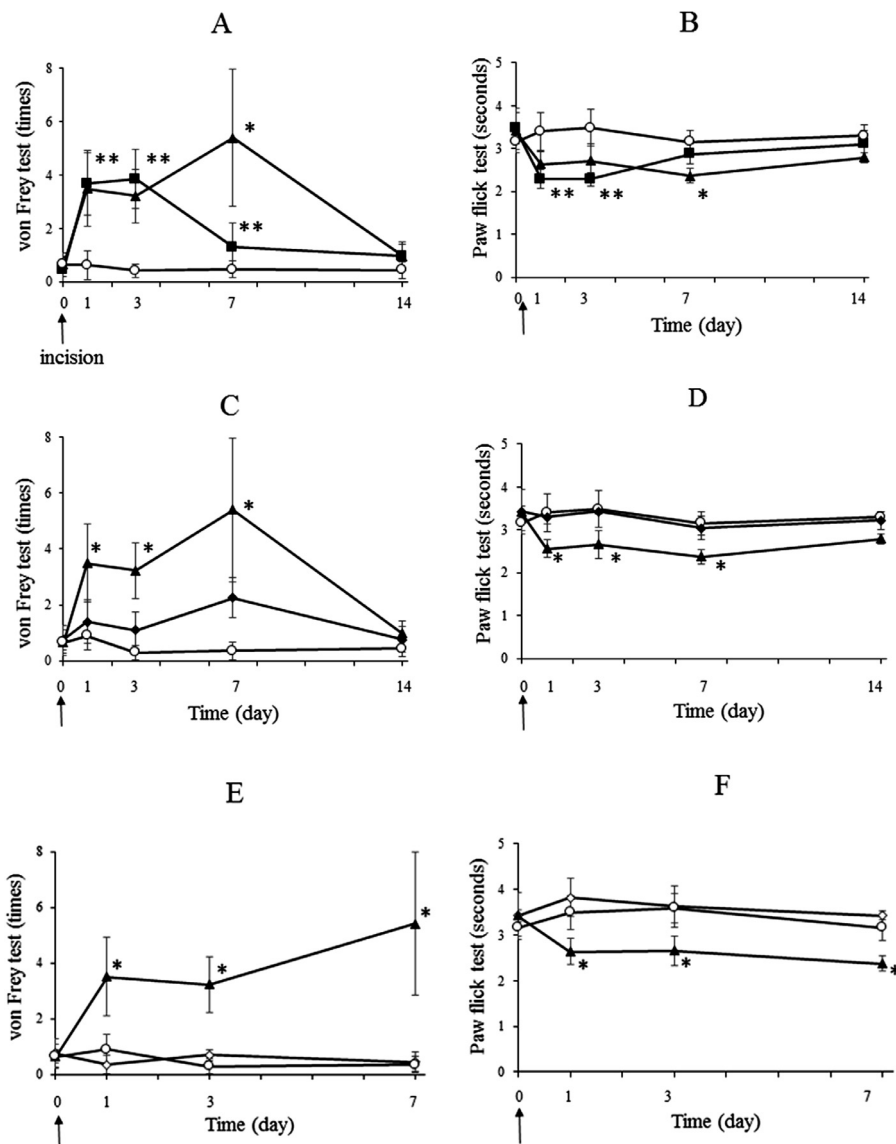
bone long term [23]. In addition, the significant improvement in pain-like behaviors was initially observed from day 7 after the start of ALN treatment. We, therefore, believe that the improvement in those behaviors by treatment with ALN was likely to reflect an im-

proved bone turnover state, although we cannot rule out the direct suppressive effects of bisphosphonate on the injured soft tissue.

Pathophysiological skeletal conditions after soft tissue invasion, such as trauma and surgical treatment, are often characterized by regional osteoporotic changes [2,24-28], the triggers of which are mainly considered to be dependent on non-weight bearing and immobilization of limbs for the treatment of those skeletal conditions. However, the mechanisms underlying those pathophysiologies remain unknown. On the basis of the current and previous studies, we consider another possible mechanism involves the injured skeletal tissue directly affecting regional bone metabolism through pathophysiological changes in the micro-environment around the bone tissue [3,10,20,21,14]. In addition, we expect that treatment with anti-osteoporosis drug such as bisphosphonate in the early phase after the skeletal injury might prevent the development of the regional osteoporotic changes in the limbs. Although further study is needed to demonstrate the significance of those pathophysiological conditions, we believe that the current study indicated a possible mechanism for the triggering of regional bone metabolic disorders accompanying tissue invasion such as trauma and surgical treatment.

The present study has several limitations. First, we did not directly measure  $H^+$  or ATP levels in the bone and injured soft tissues. Second, we did not examine changes in other molecule levels including inflammatory cytokines and noxious chemicals that could be increased in the injured tissues. Third, the tests for pain-like behaviors such as the von Frey test and paw-flick test have not



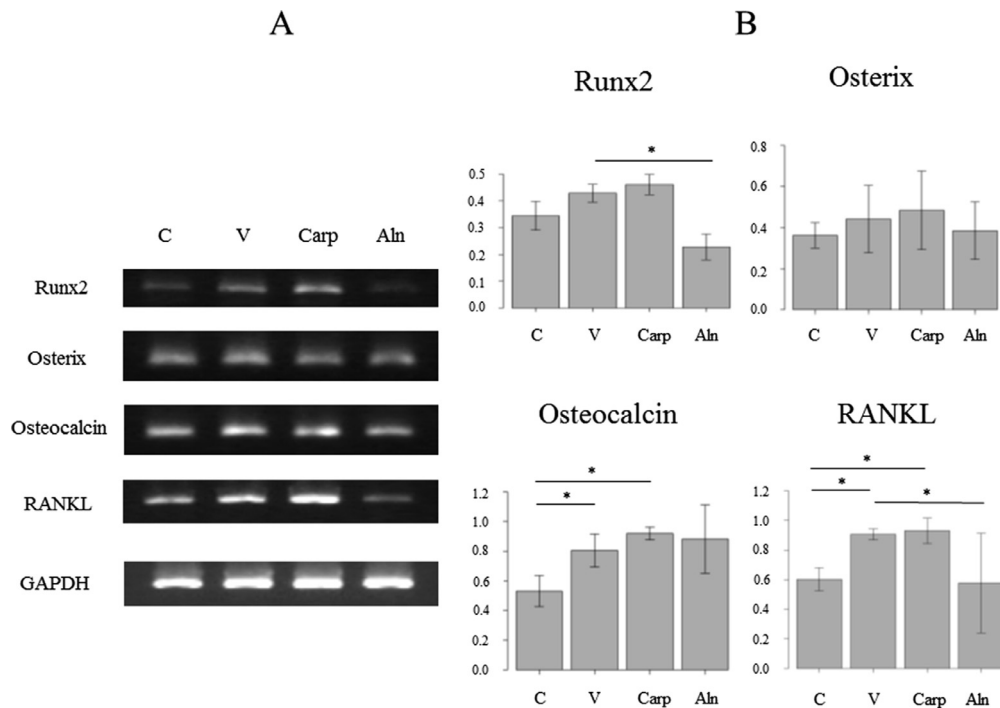


**Fig. 6.** Effects of bisphosphonate and Cox2 inhibitor (carprofen) on pain-like behaviors in the mice injured with femoral cutaneous incisions ALN treatment (black squares,  $n = 6/\text{day}$ ) significantly improved the pain-like behavior on the von Frey tests (A) and the paw-flick test (B) in the mice with femoral incisions (black triangles,  $n = 6/\text{day}$ ) at day 7 and 14. Those improvements tended to be partial in comparison with those in the limb of the non-injured mice (control; white circles,  $n = 6/\text{day}$ ) (A, B). A Cox2 inhibitor (carprofen) (black rhomboids,  $n = 6/\text{day}$ ) significantly and immediately improved the pain-like behaviors from day 1 after the start of treatment (C, D). The level of pain-like behavior on the von Frey test still tended to be higher than that in the control mice (C). Simultaneous treatment with both ALN and carprofen (white rhomboids,  $n = 6/\text{day}$ ) significantly and completely improved the pain-like behaviors on the von Frey test and the paw-flick test from day 1 after the incision (black triangles,  $n = 6/\text{day}$ ) to the same level as that in the control mice (white circles,  $n = 6/\text{day}$ ) (E, F). Tukey's post-hoc test, \* $p < 0.05$  (injured limb versus injured limb with the treatment), \*\* $p < 0.05$  (control versus injured limb with treatment).

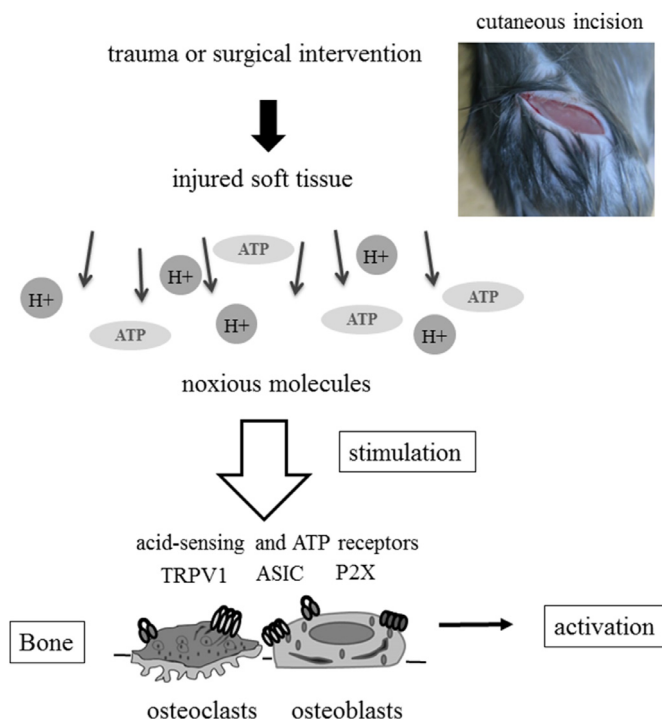
yet been established as surrogate methods for measuring skeletal pain although those tests were used for the objective and quantitative assessment of changes in animal behaviors due to bone pain in a number of previous studies [16,29–31]. Fourth, we did not demonstrate changes in bone mineral density or microarchitecture because complete wound healing of the femoral cutaneous incision took only 14 days and pathophysiological conditions of such short duration were not sufficient to affect bone microarchitecture based on our preliminary experiment (data not shown). Furthermore, the cutaneous incision model in the present study is not typical of soft tissue injury, appears to be relatively distant from clinical practice. In addition, potential infection development at open wound was not microscopically monitored, which might affect the pathophysiological changes in the injured soft tissues. In a future study, we, therefore, need to establish a new injury model

including a more severe soft tissue injury or fracture to elucidate the mechanism of regional osteoporotic changes after limb injury, and evaluate the clinical relevance of whether continuous long-term stimulation by tissue injury affects changes in bone mineral density and microarchitecture.

In conclusion, we demonstrated that, consistent with the healing process of injured soft tissue over 14 days, the expression levels of osteoblast and osteoclast differentiation regulators, including Osterix, osteocalcin and RANKL, in the femoral bone of the cutaneous-incised limbs were significantly increased up to day 7 after the incision in comparison with those in the hind limbs of control mice. The TRAP5b levels in the femoral bone and serum were also significantly increased until day 7; however, by day 14, the levels of those regulators had decreased to the same level as the control or before injury. We believe that the pathophysiological



**Fig. 7.** Effects of bisphosphonate and Cox2 inhibitor (carprofen) on the expression of osteoblast and osteoclast differentiation regulators in femoral bone at 7 days after cutaneous incision. We examined effects of ALN (Aln) and carprofen (Carp) on the expression of osteoblast and osteoclast differentiation regulators in the femur at day 7 after cutaneous incision. The ALN (Aln) significantly inhibited the increased expression level (V) of the regulators including Runx2, osteocalcin and RANKL in the femoral bone by cutaneous incision (A, B). On the other hand, the expression levels of those regulators were not significantly changed by treatment with carprofen (Carp) (A, B). Tukey's post-hoc test, \* $p < 0.05$ . C, control ( $n = 6$ ); V, vehicle ( $n = 6$ ); Carp, carprofen ( $n = 6$ ); Aln, alendronate ( $n = 6$ ).



**Fig. 8.** Hypothesis for the mechanism by which regional high bone turnover is induced by the activation of the receptors on the bone cells resulting from noxious molecule stimulation in the injured soft tissue. The increased levels of noxious molecules such as ATP and  $H^+$  under pathophysiological conditions in the injured soft tissues of limbs could affect the induction of regional bone turnover through the activation of acid-sensing and ATP receptors on the bone cells.

cal changes resulting from tissue injury associated with cutaneous incisions could be related to increases in the expressions levels of osteoblast and osteoclast differentiation regulators corresponding to high bone turnover conditions.

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**Declaration of Competing Interest**

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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