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# Different effects of Wnt/β-catenin activation and parathyroid hormone on diaphyseal and metaphyseal in the early phase of femur bone healing of mice

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## Summary

Parathyroid hormone (PTH) and agents related to the manipulation of Wnt/β-catenin signalling are two promising anabolic anti-osteoporotic therapies that have been shown to promote the healing of bone fractures. Now, it is widely accepted that cortical bone and trabecular bone are two different compartments, and should be treated as separate compartments in pathological processes, such as fracture healing. It is currently unknown whether PTH and the activation of β-catenin signalling would demonstrate different effects on cortical bone and trabecular bone healing. In the current study, single 0.6-mm cortex holes were made in the femur metaphysis and diaphysis of mice, and then, PTH application and β-catenin activation were used to observe the promoting effect on bone healing. The effects of β-catenin and PTH signalling on fracture healing were observed by X-ray and CT at 3, 6, and 14 days after fracture, and the levels of β-catenin were detected by RT-PCR assay, and the number of specific antigen-positive cells of BRDU, OCN, RUNX2 was counted by immunohistochemical staining. While β-catenin activation and PTH were found to demonstrate similar effects on accelerating metaphyseal bone healing, activation of β-catenin showed a more striking effect than PTH on promoting diaphyseal bone healing. These findings might be helpful for selecting proper medication to accelerate fracture healing of different bone compartments.

KEYWORDS

bone healing, diaphysis, metaphysis, parathyroid hormone, Wnt/β-catenin

## 1 | INTRODUCTION

Fractures are common injuries, and bone is one of the few tissues in the human body with the potential to heal completely after injury. However, there are still a small proportion of fractures that fail to heal properly, such as nonunion and malunion fractures.[1,2] In addition, the time taken for a fracture to heal is an important factor in determining a patient's recovery rate and treatment cost. Thus, therapies should not only aim to increase the chances of successful bone union but also to decrease the time required for normal fracture healing.[2,3] Many strategies have been developed to enhance or accelerate bone fracture healing, including improved surgical techniques and implant properties, bone grafts, nutrition enhancement, stem cell transplantations, and the application of growth factors.[2-6] Among these strategies, anti-osteoporosis drugs have drawn much attention since they have shown

promising effects on preventing and promoting osteoporotic fracture healing.[7-10]

Generally, anti-osteoporosis drugs can be divided into two categories: antiresorptive drugs and anabolic drugs. Bisphosphonates are the most commonly used antiresorptive drugs clinically. However, their role in the bone fracture healing process is controversial because bisphosphonates inhibit bone resorption, which is important for bone remodelling during fracture healing.[7-11] The controversy has been fuelled by case reports of a typical subtrochanteric fractures in patients receiving long-term treatment with bisphosphonates.[12,13] Denosumab is another potent inhibitor of bone resorption and would, therefore, be expected to have similar properties to the bisphosphonates.[14] The effects of selective oestrogen receptor modulators on bone repair, fracture healing, and osseointegration remain unclear. One experimental animal study in ovariectomized rats showed that raloxifene did not have an impact on the progression of fracture repair.[15] In contrast, agents with bone-forming properties would be expected to find application in the reconstruction of bone post fracture. Intermittent application of a low dose of an anabolic agent—parathyroid hormone (such as teriparatide, a recombinant human parathyroid hormone [PTH 1-34])—was shown to stimulate fracture healing and implant fixation in both animals and humans.[16-22] Agents related to the manipulation of Wnt/β-catenin signalling are another kind of anabolic drug and have attracted much attention.[1,18,23] For example, accumulating evidence supports a positive impact of neutralizing antibody against sclerostin, a Wnt/β-catenin signalling inhibitor, on fracture repair and gap defects.[18]

Cortical bone and trabecular bone are two different compartments with different structural, mechanical, and metabolic

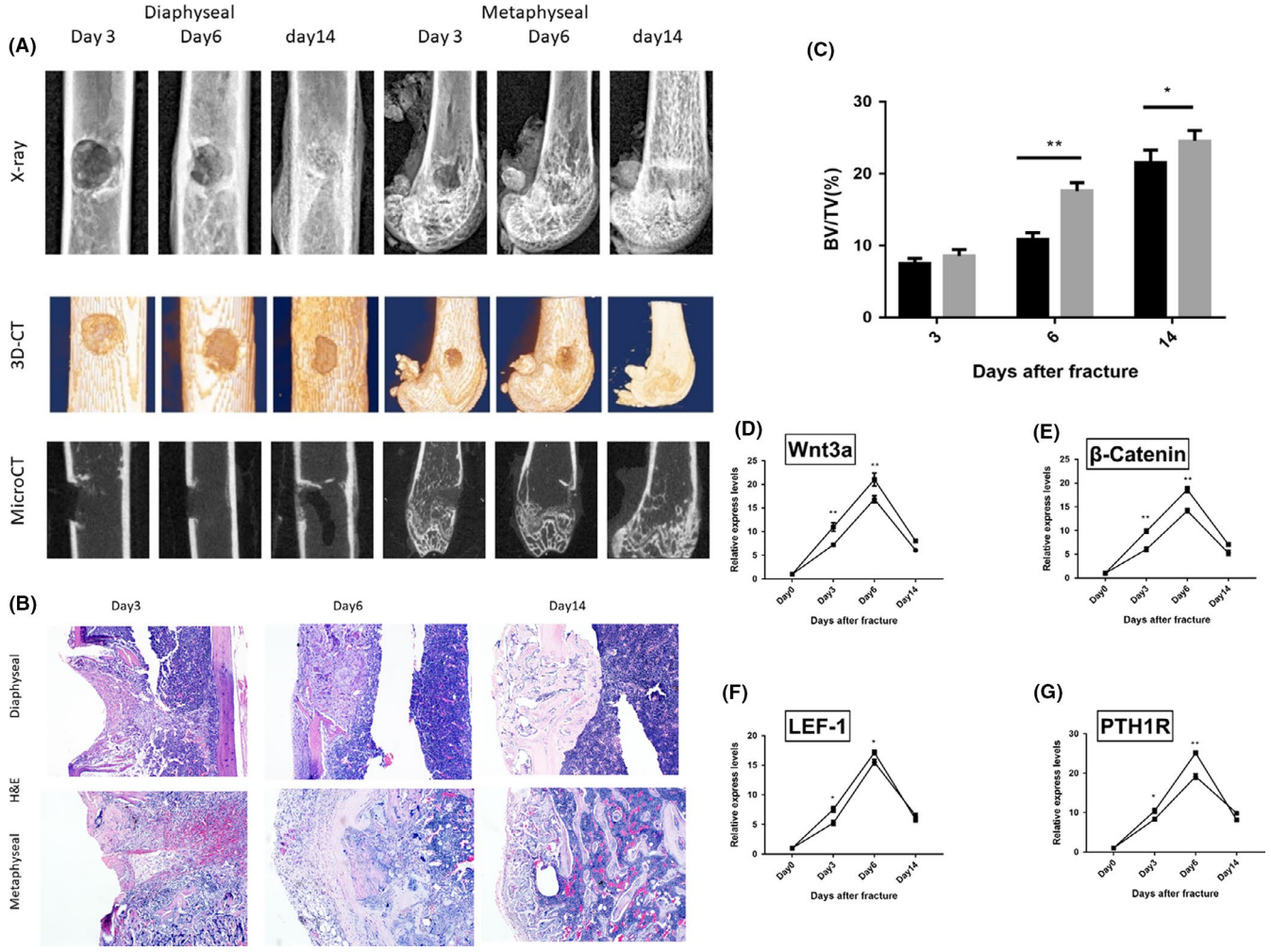


**FIGURE 1** Defect healing of femoral metaphyseal and diaphyseal in the wild-type mice. A, X-ray, 3D-CT, micro-CT images; B, H&E stainings of femurs (100×); C, bone volume/tissue volume (BV/TV) within the region of interest (ROI) were calculated. (■), WT diaphyseal; (■), WT metaphyseal; D, Wnt3a; E, β-catenin; F, LEF-1; G, PTH-1R was detected by RT-PCR. The mRNA of the fracture day was used as an internal control. The BV/TV within the ROI of metaphyseal vs that of diaphyseal, at 6 days after fracture, the $P < 0.001$, at 14 days after fracture, the $P = 0.019$. wnt3a express levels of metaphyseal vs that of diaphyseal, at 3 days after fracture, the $P < 0.001$, at 6 days after fracture, the $P = 0.002$. β-catenin express levels of metaphyseal vs that of diaphyseal, at 3 and 6 days after fracture, the $P < 0.001$. LEF-1 express levels of metaphyseal vs that of diaphyseal, at 3 days after fracture, the $P = 0.011$, at 6 days after fracture, the $P = 0.014$. PTH-1R express levels of metaphyseal vs that of diaphyseal, at 3 days after fracture, the $P = 0.011$, at 6 days after fracture, the $P < 0.001$. The results are expressed as the mean ± SD. n = 4 per group. *$P < 0.05$ and **$P < 0.01$. D-G: (–●–), Dia; (–■–), Meta

features, and they should be treated differently under many pathological conditions.[24-26] Furthermore, it was recently found that the extent of bone loss differs at different ages in humans: before 60 years of age, trabecular bone loss dominates; after 60 years of age, cortical bone loss dominates.[27,28] Therefore, it was suggested that the notion of osteoporosis as a disease of trabecular bone loss needs to be revised and that drugs having more effect on cortical bone should be developed to prevent cortical osteoporosis. This is a typical example where cortical bone and trabecular bone should be treated as two different compartments in pathological processes. It is currently unknown whether agents related to the manipulation of Wnt/β signalling and PTH, two promising anabolic therapies, would demonstrate different effects on cortical bone and trabecular bone healing. Elucidation of this issue would be helpful for drug selection for enhancing bone healing. In the present study, hole-drilling models were developed and the effects of Wnt/β-signalling activation and PTH application on bone healing were observed.

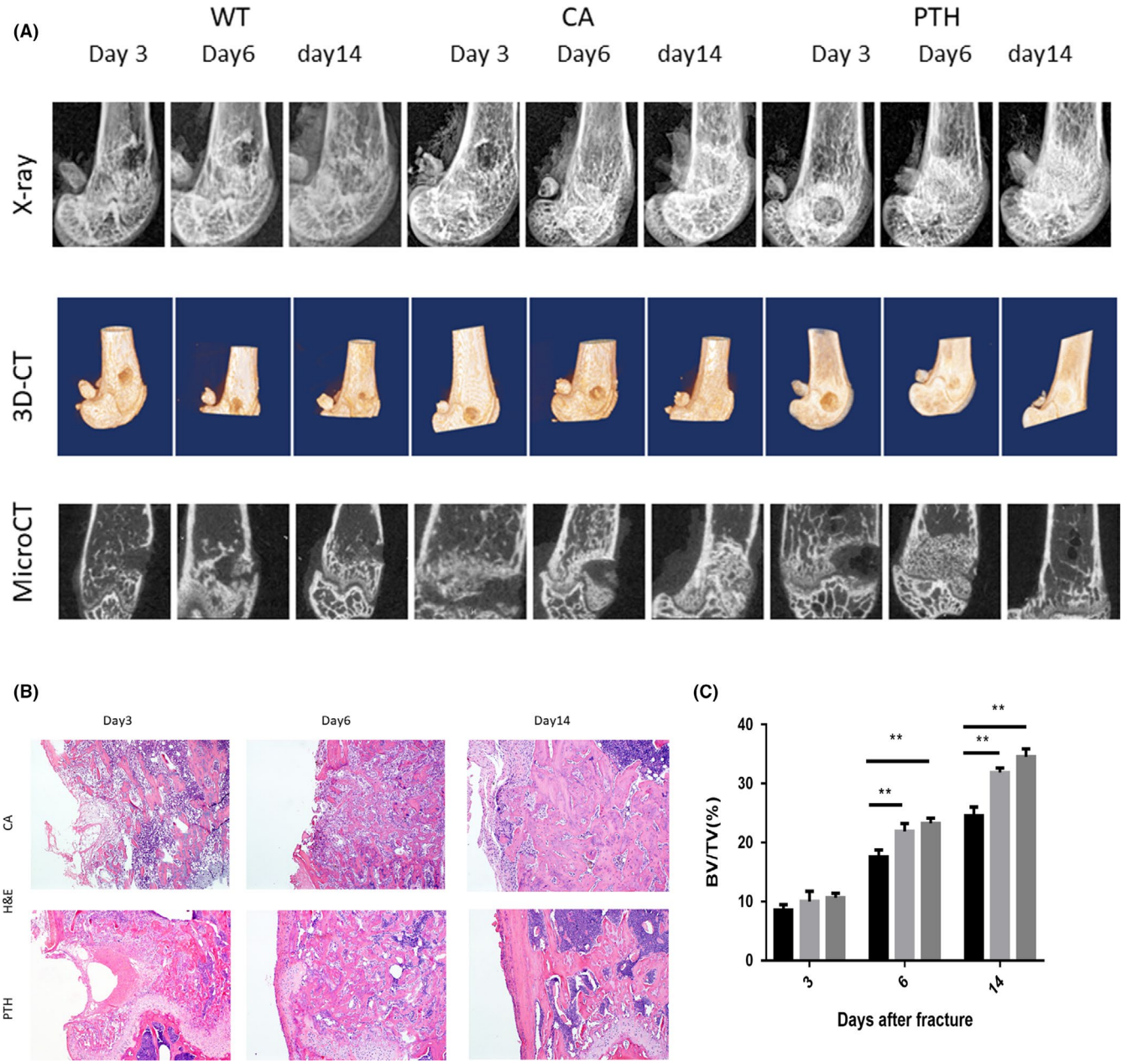


**FIGURE 2** Defect healing of femoral metaphyseal in the wild-type (WT) mice, Wnt/β-catenin activation (CA) mice and PTH activation mice. A, X-ray, 3D-CT, micro-CT images, (B) H&E staining of femurs (100×), (C) bone volume/tissue volume (BV/TV) within the region of interest (ROI) were calculated. At 6 days after fracture, BV/TV within the ROI of WT vs that of CA, and WT vs PTH, $P < 0.001$. At 14 days after fracture, BV/TV within the ROI of WT vs that of CA, and WT vs PTH, $P < 0.001$. n = 4 per group. $^{*}P < 0.05$ and $^{**}P < 0.01$. C: (■), WT; (■), CA; (■), PTH

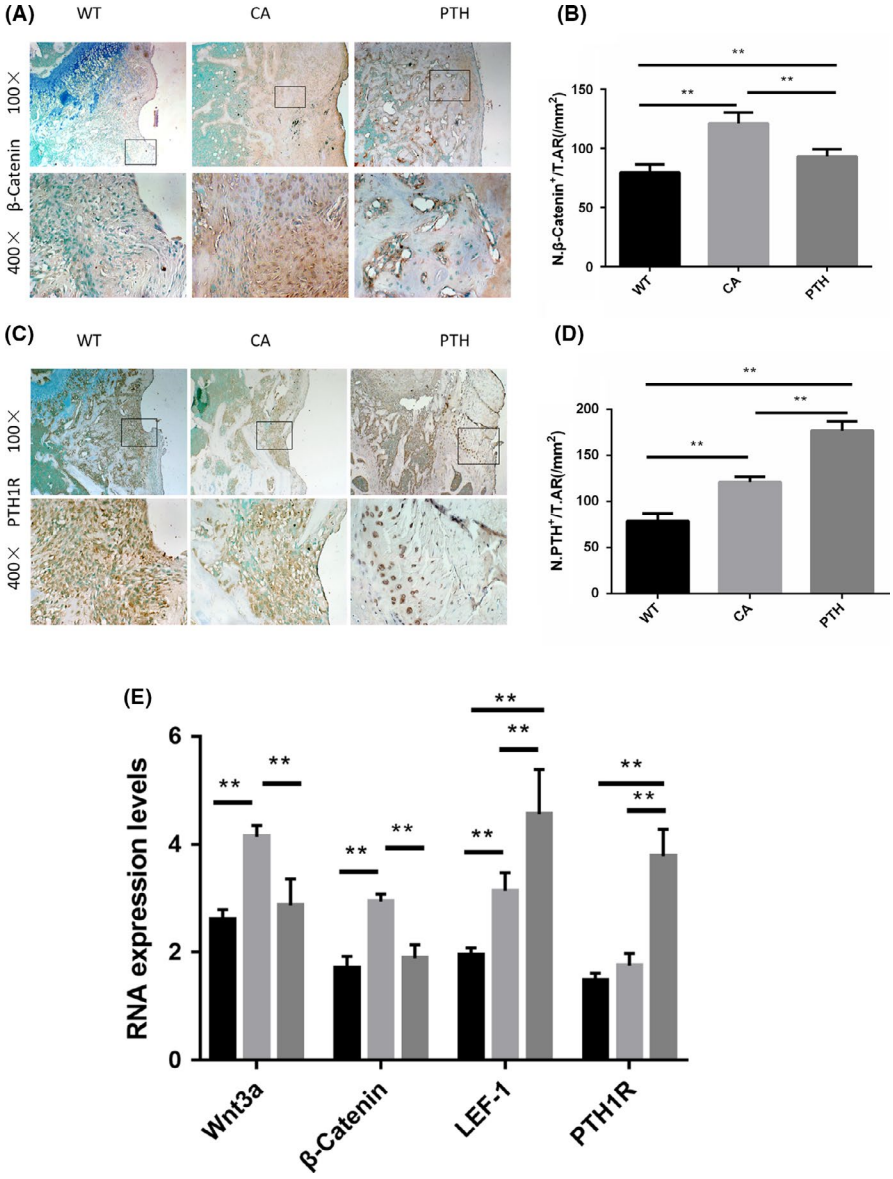


**FIGURE 3** Immunostaining for (A) β-catenin and (C) PTH1R at the defect of metaphyseal at the day 6 after fracture in the wild-type (WT) mice, Wnt/β-catenin activation (CA) mice and PTH activation mice. (B and D) Quantification of the numbers of positive cells was performed. (E) Wnt3a, β-catenin, LEF-1, PTH1R was detected by RT-PCR. The mRNA of the fracture day was used as an internal control. The numbers of β-catenin-positive cells of WT vs that of CA, $P < 0.001$, WT vs PTH, $P = 0.009$, CA vs PTH, $P < 0.001$. The numbers of PTH1R-positive cells of WT vs that of CA, WT vs PTH, CA vs PTH, $P < 0.001$. wnt3a express levels of WT vs that of CA, CA vs PTH, $P < 0.001$. β-catenin express levels of WT vs that of CA, CA vs PTH, $P < 0.001$. LEF-1 express levels of WT vs that of CA, $P = 0.009$, WT vs PTH, $P < 0.001$, CA vs PTH, $P = 0.004$. PTH1R express levels of WT vs PTH, CA vs PTH, $P < 0.001$. The results are expressed as the mean ± SD. n = 4 per group. $^{*}P < 0.05$ and $^{**}P < 0.01$. B-E: (■), WT-Met; (■), CA-Met; (■), PTH-Met

## 2 | RESULTS

### 2.1 | The speed of metaphyseal bone healing was faster than diaphyseal bone healing in wild-type mice

In WT mice, the X-ray examination and 3-D reconstruction image revealed that on day 3 after fracture, the hole in the metaphysis had become blurred (Figure 1A). The diameter of the defect had further decreased on day 6 post fracture and had disappeared by day 14 after fracture. In contrast, the hole in the diaphysis had not changed significantly by day 3 after fracture, was still clearly seen on day 6 after fracture, and had not disappeared by day 14 after fracture (Figure 1A). H&E staining demonstrated this tendency more clearly. On day 3 after fracture, the metaphyseal defect was filled with soft tissue, while the there was a large area that lacked tissue filling in the diaphyseal defect. On day 6 after fracture, there was more callus in the metaphyseal defect while the filling tissue in the diaphyseal defect was almost soft. By day 14 after fracture, complete fracture healing had occurred in the metaphyseal defect, i.e. the healing structure was the same as the normal tissue; in contrast, the diaphyseal defect had not healed fully and the defect was filled with tissue of an irregular structure (Figure 1B). Micro-CT examination showed that the BV/TV percentage was much higher in the metaphyseal defect than in the diaphyseal on days 6 and 14 after fracture. This indicates that faster bone healing occurred in the metaphyseal defect (Figure 1C).

Real-time PCR was performed to examine the mRNA expression levels of Wnt/β-catenin signalling molecules and PTH1R. The mRNA expression levels of Wnt3a, β-catenin, LEF-1, and PTH1R on days 3, 6, and 14 after fracture were higher than the day of fracture, especially on day 6 after fracture. The levels in the metaphyseal defect were higher than in the diaphyseal defect (Figure 1D-G).

These data indicate that in the current defect model, the speed of metaphyseal fracture healing was faster than diaphyseal fracture healing in wild-type mice, and that Wnt/β-catenin and PTH signalling might play a role in these effects.

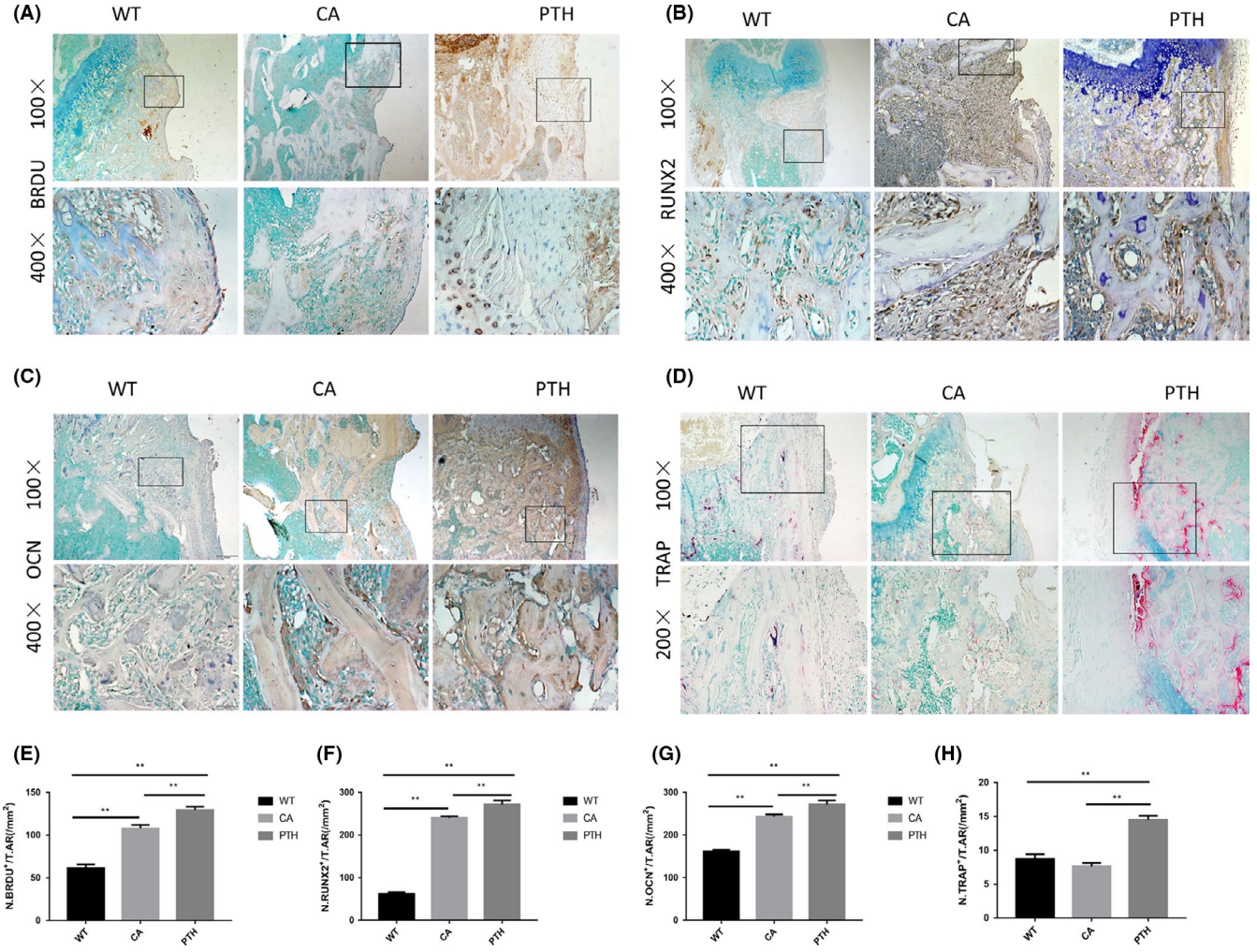


**FIGURE 4** Immunostaining for (A) BRDU, (B) RUNX2, (C) OCN and (D) TRAP staining at the defect of metaphyseal at the day 6 after fracture in the wild-type mice, Wnt/β-catenin activation (CA) mice and PTH activation mice. (E-G) Quantification of the numbers of positive cells and (H) TRAP-positive cells was performed. The numbers of BRDU-positive cells of WT vs that of CA, WT vs PTH, CA vs PTH, $P < 0.001$. The numbers of RUNX2-positive cells of WT vs that of CA, WT vs PTH, CA vs PTH, $P < 0.001$. The numbers of OCN-positive cells of WT vs that of CA, WT vs PTH, CA vs PTH, $P < 0.001$. The numbers of TRAP-positive cells of WT vs that of PTH, CA vs PTH, $P < 0.001$. The results are expressed as the mean ± SD. n = 4 per group. $^{*}P < 0.05$ and $^{**}P < 0.01$. E-H: (■), WT; (■), CA; (■), PTH

## 2.2 | Wnt/β-catenin and PTH activation showed almost effects on metaphyseal bone healing

The X-ray examination and 3-D reconstruction image revealed that the healing of the metaphyseal defect in the catenin activation (CA) group and PTH group was faster than in WT mice, especially at 6 days after fracture (Figure 2A). H&E staining showed the same tendency (Figure 2B).

As for the metaphyseal healing, the ratios of BV/TV percentage in the CA group were 1.25, 1.35, and 1.26 times greater than in the WT group at 3, 6, and 14 days after fracture, respectively. The values in the PTH group to WT mice were 1.18, 1.21, and 1.19 at 3, 6, and 14 days after fracture, respectively. That is to say, the extent of PTH was a little faster than Wnt/β-catenin activation, but without statistical significance (Figure 2C).

The number of β-catenin-positive cells in the metaphyseal defect of the CA group was greater than the WT mice and PTH group on day 6 after fracture (Figure 3A,B). It was also found that the number of PTH1R-positive cells in the metaphyseal defect of the PTH group was greater than in the WT mice and CA group on day 6 after fracture (Figure 3C,D). The CA group mRNA expression levels of Wnt3a and β-catenin were higher than that in WT mice and the PTH group on day 6 after fracture. In the PTH group, PTH1R and LEF-1 were higher than in the WT mice and CA group (Figure 3E).

During the fracture healing process, mesenchymal stem cells and osteoblast were the main reparative cells. It was found that on day 6 after fracture, the number of BrdU-positive cells (Figure 4A,E), RUNX2-positive cells (Figure 4B,F), and OCN-positive cells (Figure 4C,G) in the metaphyseal defect of the PTH group were greater than in the CA group and WT mice. However, the number of TRAP-positive cells in the metaphyseal defect of the CA group and WT mice was less than that of the PTH group (Figure 4D,H).

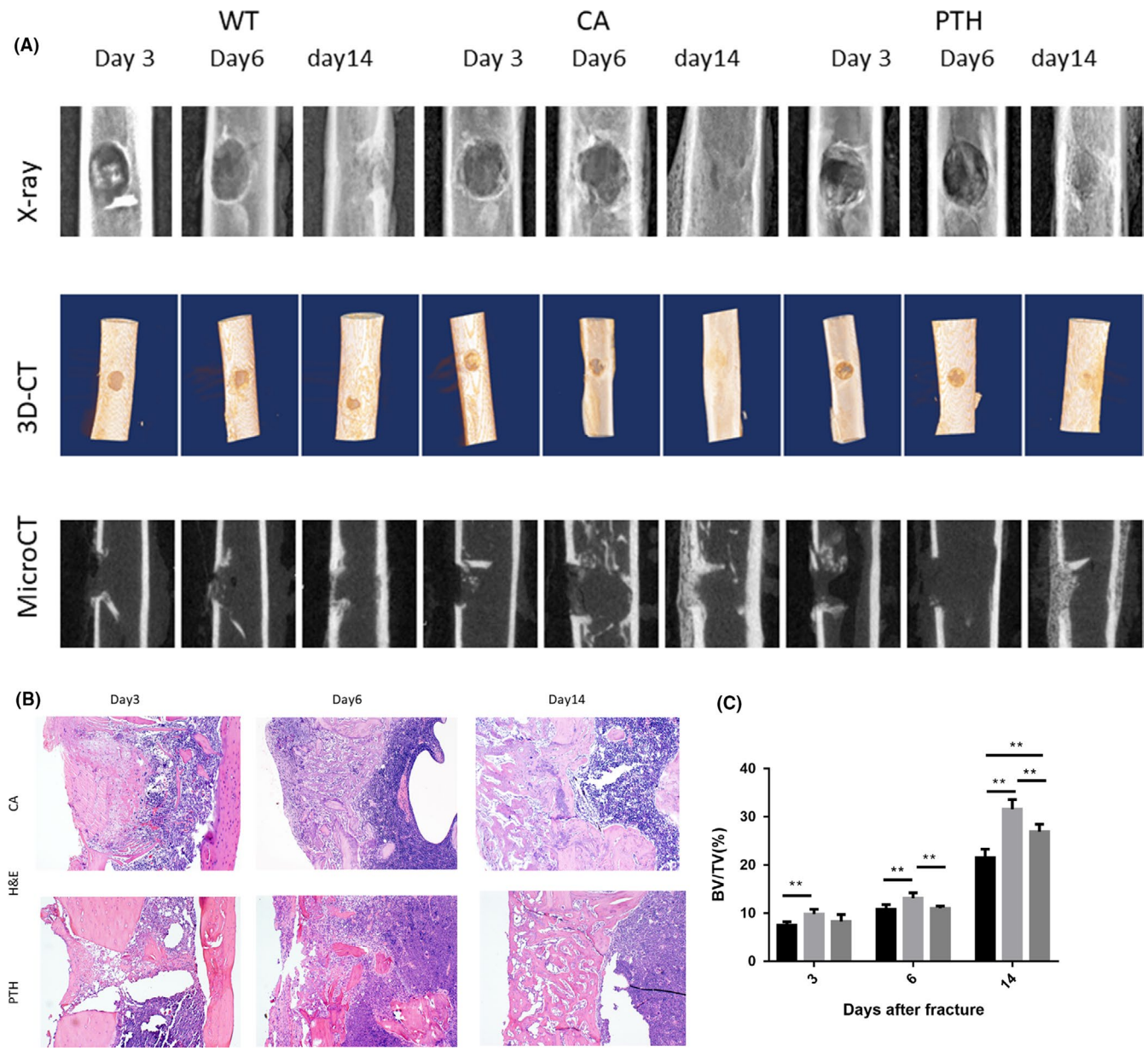


**FIGURE 5** Defect healing of femoral diaphyseal in the wild-type (WT) mice, Wnt/β-catenin activation (CA) mice and PTH activation mice. (A) X-ray, 3D-CT, micro-CT images, (B) H&E staining of femurs (100×), (C) bone volume/tissue volume (BV/TV) within the region of interest (ROI) were calculated. At 3 days after fracture, BV/TV within the ROI of WT vs that of CA, $P = 0.006$. At 6 days after fracture, BV/TV within the ROI of WT vs that of CA, $P = 0.002$, and CA vs PTH, $P = 0.003$. At 14 days after fracture, BV/TV within the ROI of WT vs that of CA, and WT vs PTH, $P < 0.001$, CA vs PTH, $P = 0.002$. n = 4 per group. $^{*}P < 0.05$ and $^{**}P < 0.01$. C: (■), WT; (■), CA; (■), PTH

## 2.3 | Wnt/β-catenin activation showed more effects than PTH activation on diaphyseal bone healing

The metaphyseal healing and the diaphyseal healing in the CA group and PTH group were faster than in WT mice, as shown by the X-ray examination and 3-D reconstruction image (Figure 5A). Therefore, we next examined whether there was a difference between the healing of cortical bone defects of CA and PTH group.

X-ray examination and 3-D reconstruction images revealed that on day 3 after fracture, the hole in the diaphysis had become more blurry in the CA group than the PTH group. The diameter of the defect in the CA group decreased further than the PTH group by day 6 post fracture. The 2-D micro-CT reconstruction and H&E staining showed the same tendency more clearly (Figure 5A,B). Micro-CT examination showed that the BV/TV percentage in the diaphyseal defect in the CA group was much higher than in the WT mice and the PTH group at all three time points. The BV/TV percentage in the diaphyseal defect in the PTH group was higher than in WT mice 14 days after fracture (Figure 5C). These results showed that the speed of the diaphyseal fracture healing in the CA group was faster than that in the PTH group at all three time points.

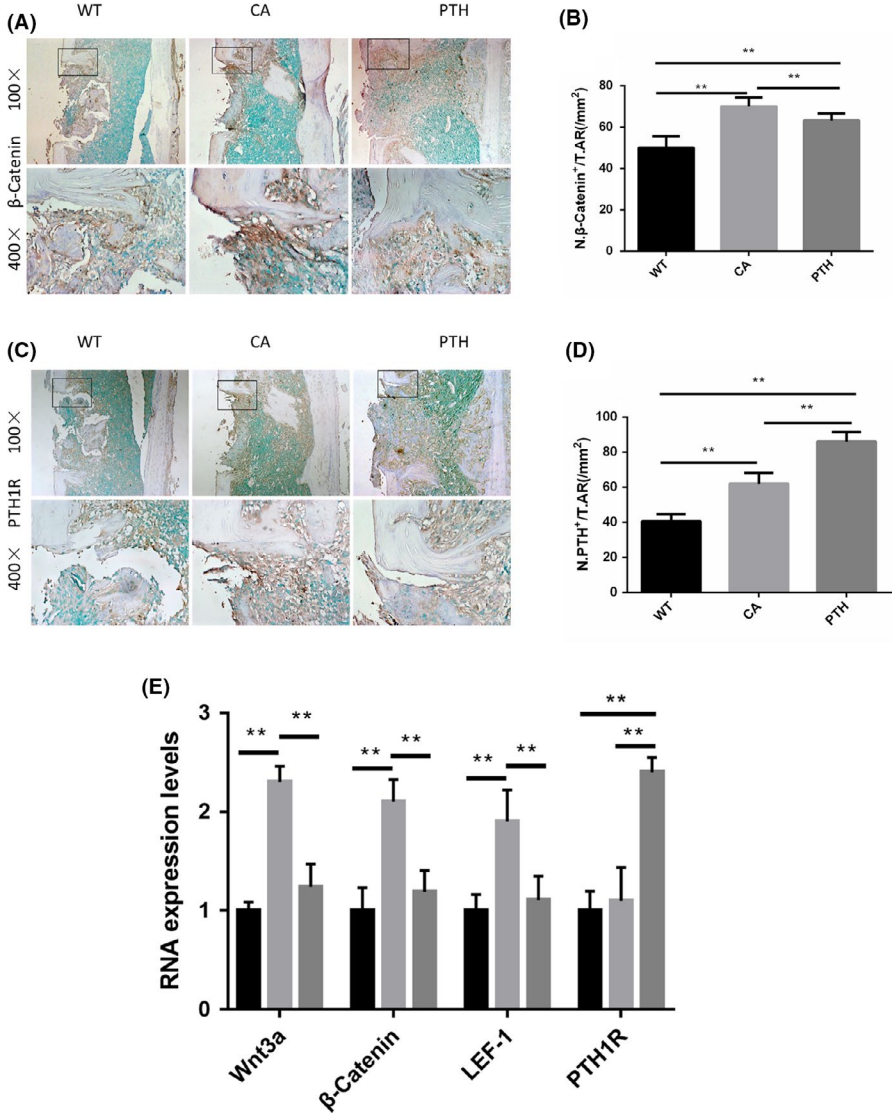


**FIGURE 6** Immunostaining for (A) β-catenin and (C) PTH1R at the defect of diaphyseal at the day 6 after fracture in the wild-type (WT) mice, Wnt/β-catenin activation (CA) mice and PTH activation mice. (B and D) Quantification of the numbers of positive cells was performed. (E) Wnt3a, β-catenin, LEF-1, PTH-1R was detected by RT-PCR. The mRNA of the fracture day was used as an internal control. The numbers of β-catenin-positive cells of WT vs that of CA, $P < 0.001$, WT vs PTH, $P = 0.001$, CA vs PTH, $P = 0.006$. The numbers of PTH1R-positive cells of WT vs that of CA, WT vs PTH, CA vs PTH, $P < 0.001$. wnt3a express levels of WT vs that of CA, CA vs PTH, $P < 0.001$. β-catenin express levels of WT vs that of CA, CA vs PTH, $P < 0.001$. LEF-1 express levels of WT vs that of CA, $P = 0.001$, CA vs PTH, $P = 0.002$. PTH1R express levels of WT vs PTH, CA vs PTH, $P < 0.001$. The results are expressed as the mean ± SD. n = 4 per group. $^{*}P < 0.05$ and $^{**}P < 0.01$. B-E: (■), WT-Dia; (■), CA-Dia; (■), PTH-Dia

More β-catenin-positive cells were found in the CA group than in the WT and PTH groups (Figure 6A,B) and more PTH1R-positive cells in the diaphyseal defect in the PTH group than the WT and CA groups (Figure 6C,D). The mRNA expression levels of Wnt3a, β-catenin, and LEF-1 in the CA group were higher than in the WT and PTH groups, and the level of PTH1R in the PTH group was higher than in the WT and CA groups on day 6 after fracture (Figure 6E).

On day 6 after fracture, the number of BrdU-positive cells in the diaphyseal defect of the CA group and PTH group was greater than in the WT group. However, there was no significant difference between the CA group and the PTH group (Figure 7A,E). The number of RUNX2-positive cells in the diaphyseal defect of the CA group was greater than the WT mice and PTH group (Figure 7B,F). OCN-positive cells in the diaphyseal defect of the PTH group were greater than in WT mice and the CA group (Figure 7C,G). The number of TRAP-positive cells in the diaphyseal defect of the CA group was less than in the WT mice and PTH group (Figure 7D,H).

These data indicate that while Wnt/β-catenin activation and PTH had similar effects on accelerating metaphyseal fracture healing, Wnt/β-catenin activation had a greater effect on promoting diaphyseal fracture healing than PTH.

## 3 | DISCUSSION

In addition to improvements in surgical techniques and implant properties, bone grafts, nutrition enhancement, and stem cells transplantations, drugs have been used to enhance bone fracture healing. Anti-osteoporosis drugs have drawn much attention since they have shown promising effects on preventing osteoporotic fracture and promoting healing. While the role of antiresorptive drugs in the process of bone fracture healing is controversial, some studies have shown that anabolic drugs might be beneficial for bone fracture healing.[7-10] Among them, PTH and an agent related to the manipulation of Wnt/β-signalling are two kinds of promising drugs. Daily treatments with 20 μg PTH (1-34) result in dose-dependent increases in bone mineral density in the lumbar spine and femoral neck in osteoporotic female and male patients. Both animal models

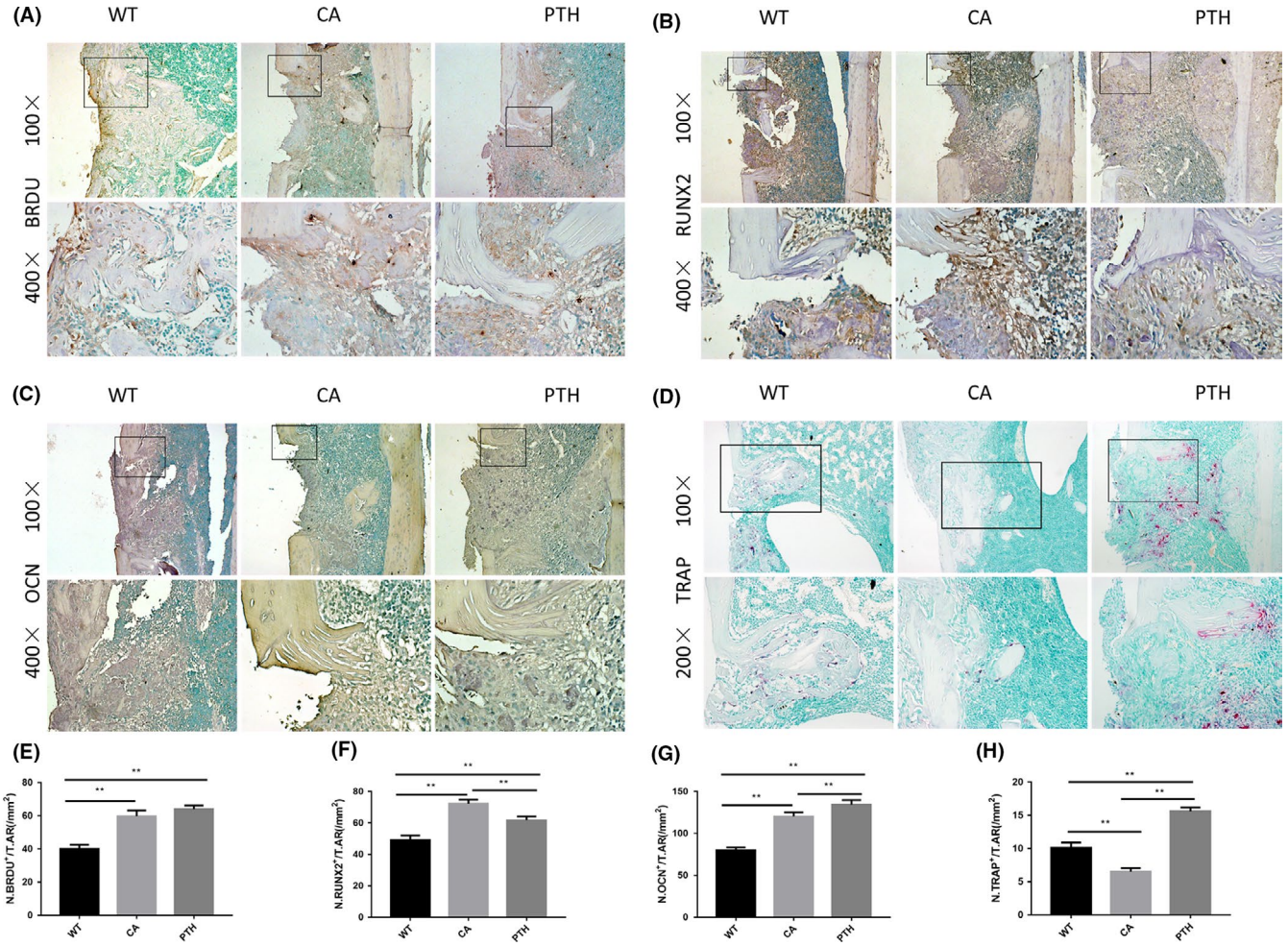


**FIGURE 7** Immunostaining for (A) BRDU, (B) RUNX2, (C) OCN and (D) TRAP staining at the defect of diaphyseal at the day 6 after fracture in the wild-type mice, Wnt/β-catenin activation (CA) mice and PTH activation mice. (E-G) Quantification of the numbers of positive cells and (H) TRAP-positive cells was performed. The numbers of BRDU-positive cells of WT vs that of CA, WT vs PTH, $P < 0.001$. The numbers of RUNX2-positive cells of WT vs that of CA, WT vs PTH, CA vs PTH, $P < 0.001$. The numbers of OCN-positive cells of WT vs that of CA, WT vs PTH, $P < 0.001$, CA vs PTH, $P = 0.002$. The numbers of TRAP-positive cells of WT vs that of PTH, WT vs PTH, CA vs PTH, $P < 0.001$. The results are expressed as the mean ± SD. n = 4 per group. $^{*}P < 0.05$ and $^{**}P < 0.01$. E-H: (■), WT; (■), CA; (■), PTH

and Randomized controlled trial have shown that PTH can promote fracture healing.[18,19,29] There is also accumulating data showing that measures related to the manipulation of Wnt/β-signalling, such as the inhibition of Glycogen synthase kinase 3 beta, activation of β-catenin, and the application of Dickkopf-1 antibody and sost antibody, promote fracture healing.[30,31]

It is now widely accepted that cortical bone and trabecular bone are two different compartments with different features, and should be treated as separate compartments in pathological processes, such as fracture healing. It is currently unknown whether agents related to the manipulation of Wnt/β-signalling and PTH would demonstrate different effects on cortical bone and trabecular bone fracture healing.

After a fracture, the Wnt signal rose and reached a peak in the middle of the fracture.[32] A related study showed that the PTH signal rose after a fracture,[33-35] similar to the changes of the related mRNA expressed after a fracture that we observed in the wild-type group, The speed of metaphyseal bone healing was faster than diaphyseal bone, Wnt/β-catenin and PTH signalling might play a role in these effects.

By employing a single-cortex, fully stabilized defect model, we directly compared the effect of activation of β-catenin and PTH on metaphyseal and diaphyseal bone healing. It was found that while activation of β-catenin and PTH demonstrated similar effects on accelerating metaphyseal bone healing, activation of β-catenin showed a more striking effect than PTH on promoting diaphyseal bone healing.

When taking only the diaphysis for analysis, it was found that β-catenin activation was more powerful than PTH in accelerating diaphyseal fracture healing, which consistent with the findings of Agholme[18] and Sandberg et al.[19] And they promoted diaphysis fracture healing better than wild group, and promoted the expression levels of OCN, RUNX2, LEF-1 in the defect, as others report.[36-38] In rat, a low dose of PTH enhanced metaphyseal repair, whereas the Scl-ab had mainly cortical bone effects with less influence on metaphyseal

healing. Therefore, new clinical studies of PTH treatment for accelerating corticocancellous fracture repair are warranted.[18] In this study, we found that the expression levels of wnt3a, catenin and LEF-1, number of β-catenin, and RUNX2 positived in the CA group were superior to those in the PTH group, while the level of RNA expression PTH1R, number of OCN positived was lower than that in the PTH group. Regional cell proliferation was not significantly different, but the osteoclasts in the CA group were significantly less than the PTH group. The β-catenin activation takes an osteoblast-promoting effect similar to PTH in the diaphyseal bone fracture region, and better ability of inhibiting osteoclasts, results in better fracture healing. Taken together, it was suggested that β-catenin activation and the related drugs, such as sost antibody, but not PTH, should be considered for accelerating diaphysis fracture healing.

As for metaphyseal fracture healing, there was no significant difference between β-catenin activation and PTH. They promoted metaphyseal fracture healing better, however, than the wild group, and promoted the expression levels of OCN, RUNX2, LEF-1 in the defect, as others report.[18,39] These results are not fully consistent with the findings of other studies that have suggested that Wnt/β-catenin does not have an effect on metaphyseal fracture healing.[18] Many other studies have demonstrated that Wnt/β-catenin has a potent effect on promoting metaphyseal fracture healing.[31,40] In addition, in a muscle-paralyzed rat model, low-dose PTH was found to improve metaphyseal bone healing.[19] Interestingly, we found that the RNA expression levels of wnt3a and β-catenin, number of β-catenin positived levels were better in the CA group than in the PTH group, while the PTH1 R and LEF-1 levels were lower than the PTH group, and the number of BRDU, RUNX2, OCN positived level was smaller than the PTH group, but the osteoclasts in the CA group were significantly less than the PTH group. Thus, it could enhance both metaphyseal fracture healing and diaphyseal healing. This may suggest that at the metaphyseal fracture healing, wnt/β-catenin does not enhance the same osteogenesis ability as PTH, but its ability to inhibit osteoclasts can makes a similar promoting results during early healing of the fracture.

Intermittent PTH increased undifferentiated Td-Tomato MSC and osteoblast number, and might transform lining cell into osteocytes and osteoblasts, and thus lead to bone formation, but there was no significant difference in osteocyte and osteoclast numbers.[41] Wnt/β-catenin activation promotes fracture healing by promoting osteoblast activity and inhibiting osteoclast activity (Figures 4,7). Mesenchymal stem cells residing close to the bone surface exhibit different activities and stronger osteogenic potential than those in the central bone marrow, the cancellous bone-rich metaphyseal region has more mesenchymal progenitors than the diaphyseal region.[42] Metaphyseal region have enough MSC to be stimulated by intermittent PTH to differentiate into osteoblasts, without care about the presence of osteoclasts. But diaphyseal region do not have so much MSC to differentiate into osteoblasts, promoting osteoblast activity and inhibiting osteoclast activity by Wnt/β-catenin activation become especially important for fracture healing.

The current study has shown that activation of β-catenin and PTH have similar effects on accelerating metaphyseal bone healing, while activation of β-catenin has a more striking effect than PTH on promoting diaphyseal in the early phase of bone healing. These findings might be helpful for selecting proper medication for treating different parts of a fracture.

## 4 | METHODS

### 4.1 | Animal surgical procedures

All animal procedures were approved by the Laboratory Animal Welfare and Ethics Committee Of the Third Military Medical University, P. R. China. All methods were carried out in accordance with approved guidelines of the institution. Mice expressing the tamoxifen (TM; Sigma-Aldrich, St. Louis, MO, USA) -inducible Cre fusion protein, Cre-ER™, under the control of the 3.2-kb mouse pro-collagen 1 promoter (3.2 kb Col1-Cre ER™) were crossed with Catnb+/flox (exon 3) mice (obtained from Jianquan Feng, Department of Biomedical Sciences, Baylor College of Dentistry). Genotyping was performed as previously reported.[15] The 3.2-kb Col1-Cre ER™/β-catenin exon 3 fx +/+ was the target in mice in which β-catenin was constitutively activated in osteoblasts by TM injection.[43,44]

Single-cortex, fully stabilized defects were made in the mid-diaphysis and metaphysis of the right femurs in all the mice. Briefly, mice were anaesthetized with 1% pelltobarbitalum natricum at a dosage of 50 mg/kg and prepared for aseptic surgery. A small incision was made above the mid-diaphysis of the femur, after which the bone was exposed with blunt dissection. A 0.6-mm diameter steel burr drill bit (Fine Science Tools # 19007-07) and an electric drill were used to induce a single-cortex defect in the mid-diaphysis of the femur. Perforations disrupted the cortical, periosteal, and endocortical surfaces and extended into the marrow but did not disrupt the opposite cortical wall. Then, a defect of the same depth and diameter (1 mm) was made in the metaphysis of the femur.[45-48] During recovery from anaesthesia, all mice were kept under heat lamps to maintain constant body temperature. The mice were given free access to food and water during the experiment and were housed three rats per cage at 21°C in a room with 12 hours of light and 12 hours of dark.

### 4.2 | Animal groups, and tissue preparation

Six-week-old male mice with a mean weight of 24.8 g (SD ± 1.4 g) with the gene background of $Catnb^{lox(ex3)}$ and wild-type (WT) male littermates were divided after surgery into three groups: WT group (mice injected with saline, n = 56), PTH group (wild-type mice injected with PTH 1-34, n = 32), and the CA group ($Catnb^{lox(ex3)}$ mice injected with tamoxifen, n = 32). PTH 1-34 (Sigma) at a concentration of 1 μg/mL was administered intraperitoneally at a dose of 80 μg/kg body weight per day after the surgery until the end of the observation period (injected 3, 6, and 14 days post-surgery) in the PTH group.[22] The same volume of saline was injected into the WT group

**TABLE 1** Primers used for RT-PCR

| Genes | Primer forward sequence (5′-3′) | Primer reverse sequence |
|---|---|---|
| Wnt3a | CCCGTGCTGGACAAAGCT | TCTGCACATGAGCGTGTCACT |
| β-catenin | ACGGTGCCGCGCCGCTTATA | TAGCCATTGTCCACGCAGCGG |
| LEF-1 | AGAACACCCCGATGACGGA | GGCATCATTATGTACCCGGAAT |
| PTH1R | AGCGAGTGCCTCAAGTTCAT | ACAGCGTCCTTCACGAAGAT |
| GAPDH | GAGAAGGCTGGGGCTCATTT | CCAATATGATTCCACCCATG |

GAPDH, glyceraldehydes 3-phosphate dehydrogenase; LEF-1, Lymphoid enhancer-binding factor 1; PTH1R, Parathyroid hormone 1 receptor; Wnt3a, Wnt family member 3A.
Shown are the details of the primers used for RT-PCR, including forward (F) and reverse (R) sequences.

at the same time points. In the CA group, TM at a dose of 100 mg/kg body weight per day was injected for four days after the surgery to activate β-catenin.[49,50] Two hours, one day, and three days before the mice in each group were killed, 5′-bromo-2-deoxyuridine (BrdU, Sigma, Arklow, Ireland) was administered at a dose of 10 μL/g body weight by intraperitoneal injection. At designated time points, four mice from the WT group (0, 3, 6, and 14 days after surgery), CA group, and PTH group (6 days after surgery) were killed by an overdose of anaesthetic for analysis of total RNA in the fracture area. The X-ray radiography was performed on another four mice from each group (3, 6, and 14 days after surgery). The femurs were removed and fixed overnight for micro-CT examination. The remaining samples were decalcified in 10% ethylenediaminetetraacetic acid. Once adequately decalcified, the samples were tissue processed, embedded in paraffin, and sectioned coronally at a thickness of 4 μm. The sections were de-paraffinized and rehydrated and then used for histological analysis and immunohistochemical staining.

### 4.3 | X-ray imaging and micro-CT examination

Bone radiographs were obtained using a Faxitron MX20. The femurs were dissected and subjected to three-dimensional micro-CT analysis using a Viva CT 40 (Scanco Medical, Bassersdorf, Switzerland), following the procedural recommendations by the American Society for Bone and Mineral Research.[21] The scanning medium was ethanol, the X-ray tube potential was 45 kVp, and the voxel size was 10 $\mu m^3$. Images were reconstructed and analyzed with EVS Beam software using a global threshold of 1400 Hounsfield units. Quantitative morphometric data were based on the region of interest as follows: a diameter of 0.8 mm was taken at the defect centre with a depth of 0.4 mm. The percentage of bone volume to total volume (BV/TV) was calculated for the bone injury sites by micro-CT.[48]

### 4.4 | Three-dimensional (3D) CT

Three-dimensional images were reconstructed from the compact CT images with density, gradation, and unsharpness processing by 3D reconstruction software (mimics19.0) on a personal computer. The CT scan around the defect and images in DICOM format were archived. Using the segmentation software (Mimics: Interactive Medical Image Control System, Version 19.0, Materialize Company, Belgium) installed on a personal computer, based on thresholding and/or the semi-automated segmentation method, the CT images were processed as 3D visualization.[51,52]

### 4.5 | Hematoxylin-eosin (H&E) staining

Hematoxylin-eosin staining was performed as described in a previous report.[43] Briefly, sections were stained in Harris hematoxylin solution for 5 minutes and differentiated in 1% acid alcohol for 30 seconds. Bluing was then performed in 0.2% ammonia water for 30 seconds. Between each step, the sections were thoroughly washed with tap water. The sections were rinsed in 95% alcohol and counterstained in eosin-phloxine solution for 30 seconds, followed by rinsing in 95% alcohol for 2 minutes, 100% alcohol for 3 minutes (twice), xylene for 2 minutes (twice), and cover-slipped with Permount.

### 4.6 | Tartrate resistant acid phosphatase (TRAP) staining

Two Coplin jars (A and B) were pre-heated to 37°C with 50 mL stock basic incubation medium (sodium acetate anhydrous (9.2 g), sodium tartrate dibasic dehydrate (11.4 g), and glacial acetic acid (2.8 mL) dissolved in 1000 mL of distilled water; pH was adjusted to 4.7–5.0 with 5 mol/L sodium hydroxide. Then, 50 μL of 2% naphthol AS-BI phosphate substrate in ethylene glycol monoethyl ether was added to jar A, into which the slides were added and incubated at 37°C for 45 minutes. A few minutes before the 45 minutes had elapsed, 1 mL of 5% pararosaniline chloride and 1 mL of 4% sodium nitrite were mixed for 30 seconds, incubated at room temperature for 2 minutes without mixing, and then transferred into jar B and mixed well. The slides from jar A were then moved into jar B without rinsing. Incubation at room temperature was done for 1-3 minutes until the colour had developed, followed by rinsing and counterstaining with methyl green for 5 minutes, dehydration, and covering with Permount. All chemicals were purchased from the Chuandong Corporation (Chongqing, China).

Pictures of each section were taken under a magnification of 200×, and the number of TRAP-positive cells was counted in five random fields. The average number and standard deviation of TRAP-positive cells were calculated and used for statistical analyses.

### 4.7 | Real-time polymerase chain reaction (RT-PCR) examination

Fresh tissue around each femur defect site was removed, and then, the metaphysis and diaphysis were separated. The bone marrow cavity was removed by centrifugation at 5000 *g* at 4°C. RNA was extracted from 1 g of the prepared metaphyseal trabecular bone and diaphyseal bone. RNA was isolated using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Real-time polymerase chain reaction (PCR) with the SYBR green detection method was used to examine the expression levels of β-catenin, Wnt3a, Lymphoid enhancer-binding factor 1 (LEF-1), and parathyroid hormone 1 receptor (PTH1R). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a control, and the expression levels of a given gene were expressed as the proportion relative to the mean GAPDH value. The primers that were used are presented in Table 1.

### 4.8 | Immunohistochemical staining

Immunohistochemistry (IHC) was performed as previously described.[53] The primary antibodies utilized were goat anti-rab Osteocalcin(OCN) (1:400), Runt-related transcription factor 2 (RUNX2) (1:200), β-catenin (1:300), and PTH1R (1:300) and goat anti-rat BrdU. The antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The biotinylated goat anti-mouse, rabbit anti-goat, and goat anti-rabbit IgGs were acquired from Boster (Wuhan, China). The percentage of cells expressing a given marker protein was obtained from photomicrographic images of each section captured with an Olympus microscope and digital camera under 400× magnification. The number of specific antigen-positive cells was counted in five random fields. The mean and standard deviation of the percentage of positive cells was calculated for each group and used for statistical analysis.

### 4.9 | Statistical analysis

All data were expressed as the mean ± standard deviation. Statistical significance was evaluated by one-way ANOVA using SPSS 11.0 software. Data were considered significant at $P < 0.05$.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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