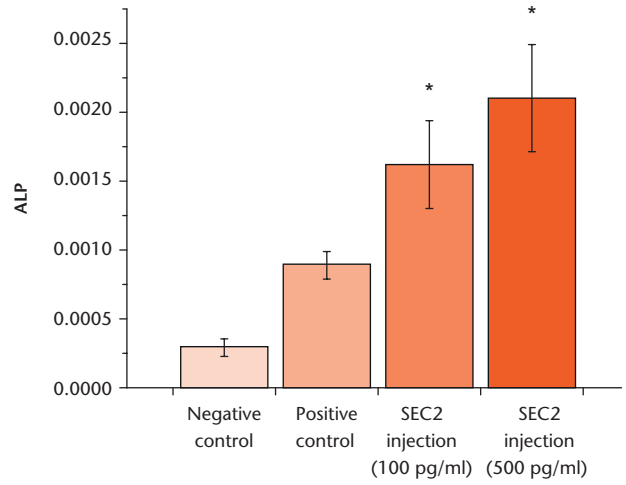


Fig. 2a

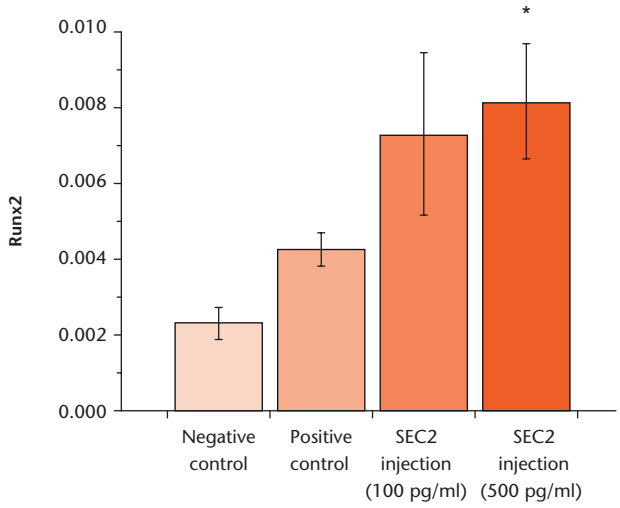


Fig. 2b

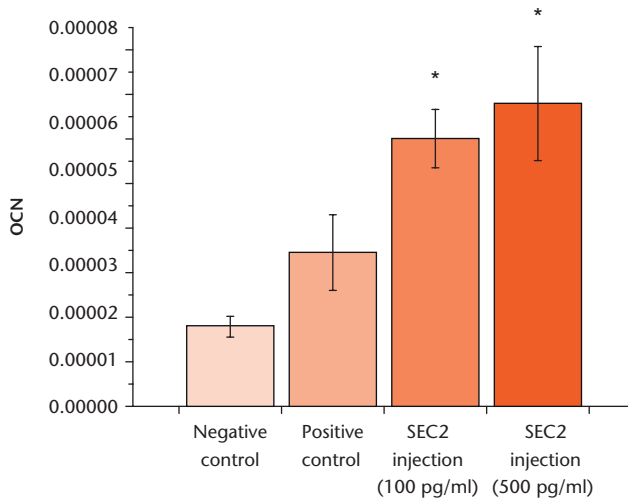


Fig. 2c

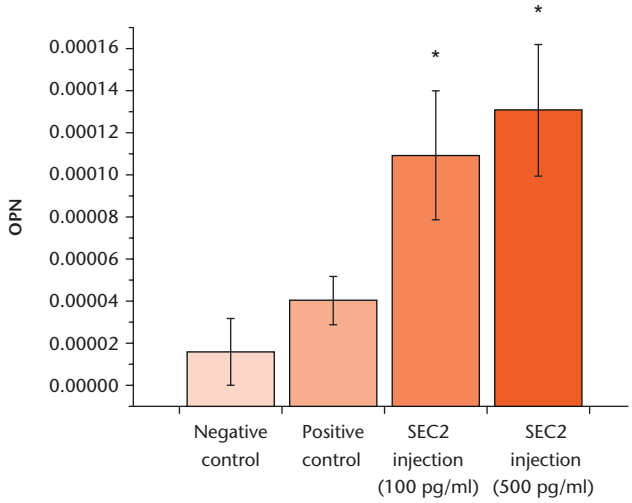


Fig. 2d

Staph lococcal enteroto in C2 (SEC2) promoted the expression of the osteogenic markers during the osteogenesis of bone marrow-derived mesenchymal stem cells (BMSCs). The osteogenic marker genes, including a) alkaline phosphatase, b) runt-related transcription factor 2, c) osteocalcin, and d) osteopontin, were examined by real-time quantitative polymerase chain reaction (qRT-PCR) assays. *$p < 0.05$ *ers s* negative control.

concentrations (1 pg/ml to 500 pg/ml) for one day, three days, and seven days, the cell viabilities were determined with MTT assays by using a Benchmark Plus microplate spectrometer (Bio-Rad Laboratories, Hercules, California).

Alizarin Red S staining. The Alizarin Red S staining was used to evaluate the calcium deposits formation. Cells were washed with phosphate buffered saline (PBS) and fixed with 75% ethanol for ten minutes, then 1% Alizarin Red S was added and maintained for 15 minutes. The result was measured at 550 nm using an automated plate reader (Thermo-Labsystems Inc., Leuven, Belgium) after elution with 1 ml of 10% cetylpyridinium chloride in 10 mM sodium phosphate.

Real-time quantitative polymerase chain reaction (qRT-PCR). After ten days' osteoinduction of BMSCs, total cellular RNAs were extracted using the RNeasy mini kit (Qiagen, Dusseldorf, Germany). The cDNAs were then reversely transcribed from the extracted RNAs by PrimeScript RT Master Mix (Takara, Kusatsu, Shiga, Japan). The Power SYBR Green PCR Master Mix (Thermo Fisher Scientific) was applied in the quantitative RT-PCR to detect the target mRNAs by using ABI 7300 Fast Real-Time PCR Systems (Applied Biosystems, Foster City, California). The primers of osteogenic genes, such as alkaline phosphatase, runt-related transcription factor 2 (Runx2) osteocalcin, and osteopontin, are listed in Table I. The relative fold changes of candidate genes were analyzed by using the $2^{-\Delta\Delta Ct}$ method.

Rat femoral fracture model. This animal experiment was approved by the Animal Research Ethics Committee. The rat femoral fracture model was performed as previously reported.[13,14] A total of 20 Sprague Dawley male rats (all 12 weeks old) were divided into two groups: treatment group (n=10) and control group (n=10). After

intraperitoneally anaesthetizing the rats with ketamine and xylazine, a mid-femoral transverse osteotomy was made with a sagittal saw. The incision was washed and closed in two layers with absorbable suture. After seven days of the operation, 0.82 ng (in 41 ul PBS) SEC2 was locally injected into the fracture site every three days (until termination) under x-ray assays. For the control group, the same volume of PBS was injected.

Radiography examination. The fracture was monitored weekly using a digital radiographic machine (Faxitron MX-20 with DC-2 option, Faxitron, Tucson, Arizona). The fractures were scored as described previously.[15] Fracture union was assessed according to the mineralized callus that bridges the fracture line (right side, one point; left side, one point; anterior side, one point; posterior side, one point). The score was estimated by two independent investigators blinded to the treatments. All rats were terminated at week four and the collected fractured femora were subjected to micro-CT examination as described previously.[16] The femora were scanned by vivaCT 40 (SCANCO Medical, Bruttisellen, Switzerland) with a resolution of 10.5 µm. The scan range included 3 mm proximal and 3 mm distal to the fracture line. All samples were analyzed by using the same parameters according to our established evaluation protocol (sigma = 1.2, two voxel widths, low attenuation = 124; high attenuation = 256, in per mille of maximal image gray value). The low-density tissues represented the newly formed calluses and the high-density tissues represented the newly formed highly mineralized calluses. The following morphometric analysis parameters were accessed: TV (total volume of tissue), BV_{low} (volume of low radio-opacity bone), BV_{high} (volume of high radio-opacity bone), and total connected density. These parameters were applied to calculate the percentage of the total tissue volume.

Biomechanical testing. The femora from each group (n = 6) were harvested for four-point bending mechanical test after micro-CT examination. A four-point bending device (H25KS; Hounsield Test Equipment Ltd. UK) with a constant displacement rate of 5 mm/min was used in accordance with our previous report.[17] The femora were loaded in the anterior-posterior direction Tw -1(v;inner span blades set as 8

Control

Fig. 4a

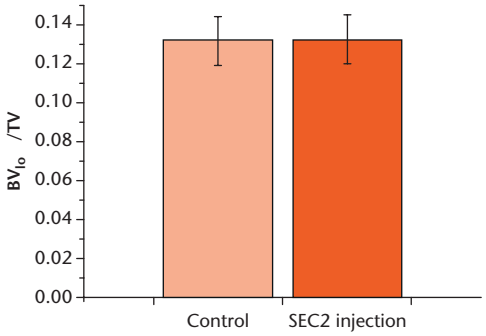


Fig. 4c

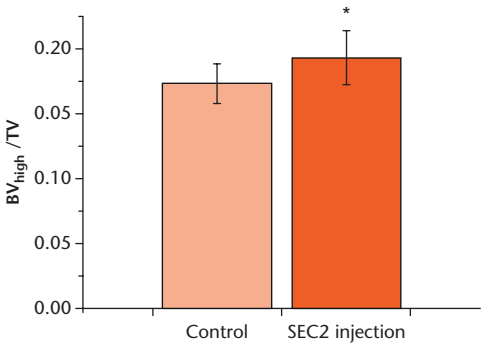


Fig. 4d

SEC2 injection

Fig. 4b

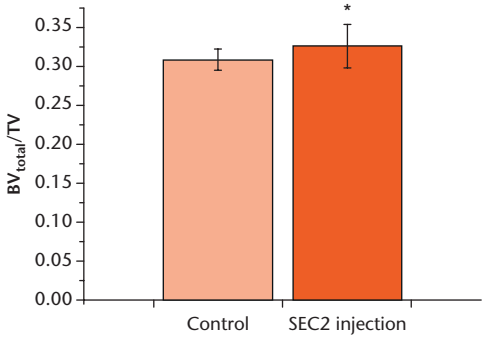


Fig. 4e

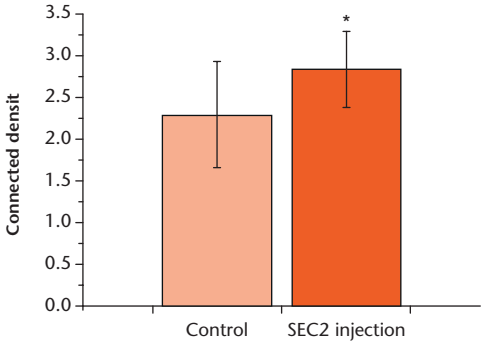


Fig. 4f

Micro-CT analyses of femora from *Staph lococcal enteroto in* C2 (SEC2) and control groups. a) and b) The representative image of micro-CT examination at four weeks. Graphs showing quantitative analyses of c) BV_{low}/TV value; d) BV_{high}/TV; e) BV_{total}/TV; and f) connected density. *$p < 0.05$ *ers s* control. BV, bone volume; TV, total volume.

sample were stained with haematoxylin and eosin, Safranin O, or immunohistochemical staining of osteocalcin for histology examinations.

Statistical analysis. Differences between groups were examined for statistical significance using analysis of variance (ANOVA), and the results were expressed as means ± sd. All experiments were performed in triplicate. The significance level was set at $p < 0.05$ (two-tailed).

Results

Effects of SEC2 on cell viability of BMSCs. BMSCs were treated with SEC2 and the effects of SEC2 on cell viability were examined. As showed in Figure 1a, no obvious effect on cell proliferation was observed with SEC2 treatment at different concentration from 1 pg/ml to 500 pg/ml.

SEC2 promoted osteogenic differentiation of BMSCs. The rat BMSCs were cultured in an osteoinduction medium with either 100 pg/ml or 500 pg/ml SEC2, and the calcium nodule formation was evaluated by Alizarin Red S staining. The results showed that SEC2 significantly enhanced the calcium nodule formation (Fig. 1b), and the increased mineralization was further quantified by colorimetric assays (Fig. 1c). We further examined the expression of osteogenic markers, such as alkaline phosphatase, Runx2, osteocalcin, and osteopontin; the results showed that these markers were significantly upregulated by both 100 pg/ml and 500 pg/ml SEC2 (Fig. 2). These results suggest that SEC2 could stimulate osteogenic differentiation of BMSCs.

Radiographic analyses of bone fracture in the SEC2-treated group. To examine the *in i o* effect of SEC2 on fracture, a rat femoral fracture model was applied in this study. SEC2 was locally injected every three days and x-ray assays were examined weekly. Although it caused a slight fever in rats, no other obvious side effects were found.[18] As shown in Figure 3a, there was no difference between groups at week one; however, the size of callus (callus width and callus area) was increased in the SEC2-treated group from week two. We also found that fracture lines remained clear in the control group at week three and four; whereas it nearly disappeared and a larger size of callus bridging the fracture gap was observed in the SEC2-treated group (Figs 3a and 3b). At week four, the mean scores of fractures union were evaluated, and the results showed that the SEC2-treated group had a significantly higher score (Fig. 3c).

Micro-CT analyses of femora from the SEC2 and control groups. To evaluate the osteoinduction ability of SEC2, micro-CT analysis was employed to quantify the newly formed bone tissues. As shown in Figures 4a and 4b, more newly mineralized calluses were observed in the the SEC2 group. Although the rats treated with SEC2 displayed no obvious increase in Bone Volume Density (low) (BV_{low}/TV) (Fig. 4c), a significant increase was observed in Bone Volume Density (high) (BV_{high}/TV) (Fig. 4d), total

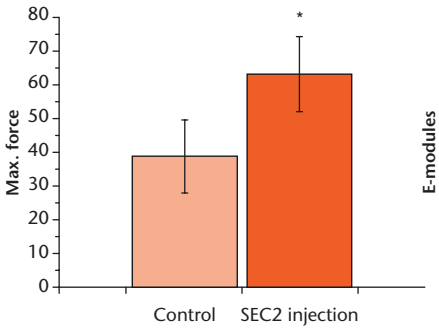


Fig. 5a

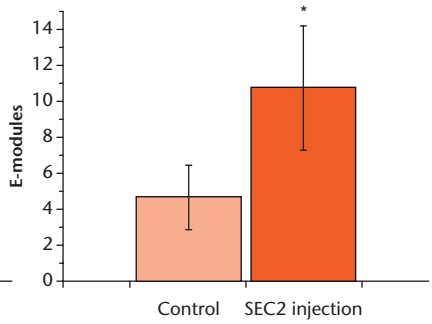


Fig. 5b

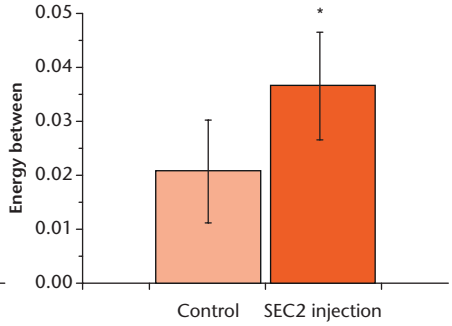


Fig. 5c

Graphs showing the mechanical testing of the repaired bones with control and *Staph lococcal enteroto in* C2 (SEC2) treatment. Fractured femora were collected for mechanical testing. Significant increases were found in a) maximum force of failure, b) modulus of elasticity, and c) energy to failure in SEC2 group. *$p < 0.05$ *ers s* control.

bone volume density (BV_{total}/TV) (Fig. 4e), and connected density (Fig. 4e). Therefore, local administration of SEC2 could enhance the healing of bone fractures.

Biomechanical testing of repaired bone with SEC2 treatment. The mechanical properties of the repaired bones could be examined by the mechanical testing. The modulus of elasticity (E-modulus), ultimate loading, and energy to failure were examined; these results indicated that they were all significantly improved in the SCE2 treated group (Figs 5a to 5c), suggesting a better biomechanical recovery of the treated bone.

Histological analyses of regenerated bone tissues. Haematoxylin and eosin staining and Safranin O staining were performed to evaluate the newly mineralized-bone tissue. As shown in Figs 6a and 6d, better callus formation was exhibited and more chondroid tissues were observed in the SEC2-injected group, suggesting that this group has greater endochondral ossification (Figs 6b and 6e). The increased osteocalcin expression was also observed in the SEC2-treated group (Figs 6c and 6f). Moreover, there was a higher percentage of chondrocytes in the uncalcified callus (Fig. 6h) and more periosteal woven bones were formed in the SEC2-treated group (Fig. 6g).

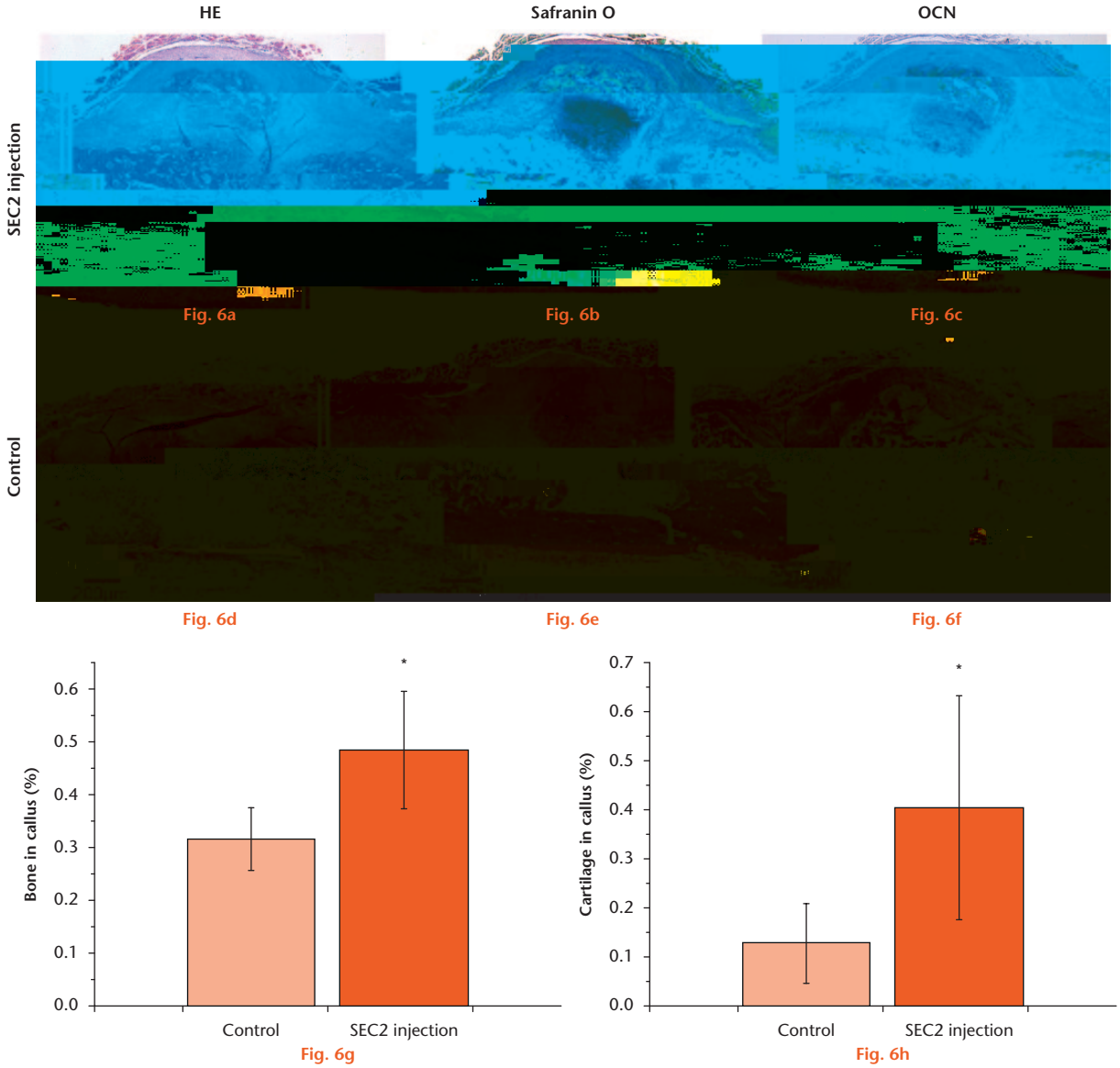
Discussion

BMSCs are multipotent cells that can be easily expanded and differentiate into multiple cell types.[19,20] It has been shown that SEC2 inhibited tumour cell growth by 60% at the concentration of 10 ug/ml.[10] In our previous study,[12] SEC2 with higher concentrations (20 ug/ml to 100 ug/ml) slightly suppressed the cell proliferation of human BMSCs. However, MTT results showed that SEC2 at lower concentrations had no effect on cell proliferation, suggesting that SEC2 has no toxicity to BMSCs.

To further confirm the effect of SEC2 on osteogenic differentiation of rat BMSCs, we examined the formation of calcium deposits and the expression of osteogenesis-related genes. As an important functional indicator of osteogenesis,[21] SEC2 could enhance the formation of calcium nodule. Upregulation of osteogenesis-related genes induced by SEC2, such as alkaline phosphatase, osteocalcin, osteopontin, and Runx2, have further demonstrated the promoting effects of SEC2 on osteogenic differentiation of BMSCs. Among these osteogenic genes, alkaline phosphatase is considered as an early osteoblast marker and osteocalcin acts as a late-stage marker of osteogenic differentiation and mineralization.[22,23] A low dose of SEC2 treatment could enhance the expression of osteopontin, a gene for prominent bone matrix protein.[12] Runx2 is a pivotal transcriptional regulator that inhibits adipogenic differentiation through blocking PPARγ2 activity.[24,25] Previously, we reported that 20 µg/ml SEC2 protein significantly increased osteogenic differentiation and inhibited osteoclast differentiation.[12] In the present study, even a lower dose of SEC2 protein (100 pg/ml) could also demonstrate a similar pro-osteogenic effect on rat BMSCs.

In addition, we further investigated the effect of SEC2 on fracture healing. Transverse fracture of long bone diaphysis is one of the most common types of fractures.[26] Therefore, an open transverse femoral (osteotomy) fracture was created in the present study and fixed using a intramedullary needle fixation. Compared with the closed fracture model, the open fracture model allows for a better controlled and more uniform fracture pattern. The results showed that local administration of SEC2 increased callus formation and improved mechanical properties.

During fracture healing, the newly formed callus consists of a less mineralized bone with a larger amount of haematoma, collagen fibers, and cartilage. The micro-CT examination was employed to analyze the bone quality. The rate of radio-opacity bone volume (BV) to total volume (TV) and the connectivity density was examined to indicate the callus formation and the mineralization. The total volume includes prefracture cortical bone, newly formed bone, and unmineralized tissues. Both BV_{low} and BV_{high} are used for monitoring the newly formed bone, while BV_{high} only represents highly mineralized callus bone. No difference was observed in BV_{low}/TV between



Histological analyses of regenerated bone tissues. The calluses were examined by a) and d) haematoxylin and eosin staining (HE), and b) and e) Safranin O staining. c) and f) The expression of osteocalcin (OCN) was examined by immunohistochemical staining. g) Graphs showing that a higher percentage of bones and h) more chondrocytes were found in *Staph lococcal enteroto in* C2 (SEC2) group. *$p < 0.05$ *ers s* control.

the two groups, which suggests their newly formed calluses remain similar. Moreover, SEC2 group exhibited more callus mineralization and remodelling, with a significant increase in BV_{high}/TV and BV_{total}/TV values. These findings revealed that SEC2 accelerated ossification during fracture healing. Furthermore, biomechanical testing also showed a better biomechanical recovery of the defected bone in SEC2 group.

On the other hand, the regenerated bone tissues induced by SEC2 were examined by histological analyses. By haematoxylin and eosin staining and Safranin O staining, the larger callus was observed in SEC2 group, indicating that the treatment group had a higher percentage of cartilage and bone tissues. These findings also suggest that SEC2 treatment could accelerate endochondral ossification. The promoted endochondral ossification was also reported with other anabolic bioactive factors such as VEGF and insulin-like growth factor-I.[27,28] Osteocalcin is mainly expressed in osteoblasts and hypertrophic chondrocytes during fracture healing.[29,30] In this study, we found an increased osteocalcin expression in the SEC2 group. Therefore, SEC2 may accelerate endochondral bone formation and the bone remodelling process. As for the molecular mechanism, although several signalling pathways, such as PI3K/mTOR signaling, NF-κB signalling, and Ca2+/calcineurin (CaN)/nuclear factor of activated T cells (NFAT) signalling, were demonstrated to be involved in SEC2-induced immune activation,[31,32] the pathway of SEC2 on osteogenesis differentiation remains elusive. As a coactivator

of Runx2/Cbfa1, the Interferon (I