

1 **Histochemical analysis of osteoclast and osteoblast distributions on**
2 **hydroxyapatite/collagen bone-like nanocomposite embedded in rat tibiae**

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16 **Running title: *Bone substitution using hydroxyapatite and collagen***
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34

35 **Abstract**

36

37 *Objectives:* Hydroxyapatite (HAp)/collagen (Col) cylinders with laminated collagen layers were
38 implanted into the tibial diaphysis of rats and examined histochemically to clarify how the orientation
39 of HAp and Col bone-like nanocomposite fibers in HAp/Col blocks affects bone resorption and
40 formation.

41

42 *Methods:* HAp/Col fibers were synthesized and compressed into cylindrical blocks to mimic bone
43 nanostructures. These were implanted into the cortical bone cavities of 10-week-old male Wistar rats
44 with fiber bundles parallel to the tibial surface. The implants were histologically analyzed at 3, 5, 7,
45 14, and 28 days after implantation.

46

47 *Results:* TRAP-positive osteoclasts appeared after 3–5 days in the lateral region of the graft, where the
48 fiber ends were exposed, but not in the bottom region, where the HAp/Col fibers were parallel to the
49 surface. Osteoclasts were observed in both regions by day 14. PHOSPHO1-positive osteoblasts were
50 first detected on day 5, appearing slightly away from the cylinder laterally but directly on the bottom
51 surface. A few osteoblasts contacted the block laterally, whereas many were observed on the new bone
52 tissue at the bottom, between days 7 and 14. Bone formation was induced earlier in the bottom region,
53 whereas lateral resorption was dominant. This suggested the uncoupling of bone resorption and
54 formation in the early postimplantation stages. However, bone remodeling shifted to coupling between
55 osteoclasts and osteoblasts throughout the cylinder by day 28.

56

57 *Conclusion:* The orientation of HAp/Col fibers in HAp/Col graft materials substantially affected the
58 preferential induction of bone resorption or formation during the early stages of bone regeneration.

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61 **Key words:** HAp/Col, bone regeneration, osteoblast, osteoclast, histochemistry

62

63 1. Introduction

64

65 Calcium phosphate-based materials have long been used as bone substitute materials for grafts. Bone
66 substitutes containing collagen fibers in addition to calcium phosphate have been developed to more
67 closely mimic the properties of the bone matrix *in vivo*. However, the characteristics of the calcium
68 phosphate components have been seen as the primary factors influencing the success of bone
69 regeneration following grafting, while collagen fibers have been considered auxiliary, contributing
70 mainly to cell adhesion and migration.

71 *In vivo*, the bone matrix consists of a 1:1 hydroxyapatite (HAp) to collagen (Col) volume ratio,
72 corresponding to an approximate 73:27 mass ratio, which suggests that collagen fibers play a
73 significant role in bone formation and regeneration. The collagen fiber arrangement differs
74 significantly between mature cortical bone and immature bone tissues, such as primary trabeculae [1].
75 Therefore, it is crucial to consider the geometric arrangement of collagen fibers when evaluating bone
76 regeneration using calcium phosphate-based bone grafts containing collagen fibers.

77 The degree of mineralization of collagen fibers in the bone matrix may depend on the individual
78 age and the position of bone in the body. Immature bone, such as the primary trabeculae beneath the
79 growth plate cartilage during development and growth, exhibits a histologically immature structure
80 with randomly dispersed collagen fibrils [1], lacking a lamellar bone structure. In contrast, cortical
81 bone, which forms the thick outer wall of long bones, exhibits a lamellar structure with bundles of
82 collagen fibers regularly arranged in the lamellar layers [2]. In addition, the *c*-axes of HAp nanocrystals
83 align along the long-axes of collagen molecules, forming the mineral surface of bone, *i.e.*, the *m*-
84 surface (This is often called the *a*-surface, but the crystallographically correct name is *m*-surface;
85 therefore, in this paper, the authors use “*m*-surface”) [3]. Nakano et al. [4] also demonstrated this
86 arrangement using X-ray diffraction analysis. Moreover, Hasegawa et al. [5] revealed that HAp crystals,
87 which are needle-like in shape and similar in length to the periodic banding of collagen fibers, are
88 consistently and precisely aligned along the collagen fibers. Thus, HAp crystal arrangement, along
89 with collagen fiber arrangement, may also affect bone formation and regeneration.

90 In cortical bone, collagen bundles form layered structures with parallel fibers, creating a
91 plywood-like arrangement of stacked lamellae [2]. The HAp/Col bone substitute developed by Kikuchi
92 et al. [6] closely mimics this structure, although the collagen fibers are not unidirectional in two
93 dimensions within each layer. This differs from other calcium phosphate-based bone substitutes with
94 collagen fibrils, such as Bio-Oss® (Geistlich Pharma AG, Switzerland), Collagraft® (Zimmer Biomet),
95 and BONEJECT® (GC Corporation), which have different compositions and structures [7,8].
96 Additionally, the HAp/Col composite can be fabricated in dense, porous, and sheet-like forms [6,9,10].

97 Therefore, it is possible to laminate layers of HAp/Col to form a HAp/Col cylinder with a laminate
98 structure consisting of horizontally aligned collagen fibers in stacked sheets.

99 In this study, by embedding layered HAp/Col cylinders with aligned collagen fiber bundles into
100 the cortical bone of rat tibiae, we examine whether collagen fiber orientation influences the distribution
101 of osteoclasts and osteoblasts in regions where the fibers run perpendicular or parallel to the bone
102 structure and assess new bone formation during bone regeneration.

103

104 **2. Materials and Methods**

105

106 *2.1. Preparation of the HAp/Col block for bone substitute*

107 Water for Injection (Otsuka Pharmaceutical, Japan) was used in HAp/Col synthesis to avoid
108 contamination of endotoxins. The HAp/Col bone-like nanocomposite fibers were synthesized via the
109 previously reported simultaneous titration method [6]. Briefly, 199.1 mmol of Ca(OH)₂ (prepared by
110 hydrating CaO obtained from the heat decomposition of alkaline analysis grade CaCO₃ [Fuji Film
111 Wako Pure Chemical, Ind]) dispersed in 2 dm³ of H₂O and a 59.7 mM H₃PO₄ (Fuji Film Wako Pure
112 Chemical, Ind., Japan) aqueous solution containing 5 g of atelocollagen (derived from porcine dermis,
113 Nitta Gelatin, Inc., Japan) were simultaneously and gradually added through respective tube pumps
114 into a central reaction vessel containing 1 dm³ of H₂O. The starting materials were prepared so that the
115 final composite would have a HAp:collagen mass ratio of 80:20. A 40°C water bath controlled the
116 reaction temperature, and the reaction solution pH was maintained at 9.0 ± 0.1 by an automatic titration
117 unit. The precipitates were filtrated with suction and washed 3 times with H₂O.

118 To maintain a lamellar fiber structure, a HAp/Col disk was prepared by packing 32.6 g of the
119 HAp/Col fibrous precipitate into a specially designed mold allowing excess water removal and
120 uniaxially compressing it at 20 MPa for 24 h, forming a 35 mm-diameter, 3.64 mm-thick disk. Three-
121 millimeter-diameter HAp/Col cylinders were punched out of the disk with a 3 mm punch for leather,
122 dried in a vacuum, and sterilized with ethylene oxide gas.

123

124 *2.2. Animals and the preparation of bone defects and grafts*

125 The HAp/Col cylinders were implanted into the anterior region of the tibia of rats (Fig. 1). Thirty 10-
126 week-old male Wistar rats (Japan SLC Co. Inc., Japan) were handled according to Hokkaido
127 University's guidelines for animal care and research use (approved study protocol #23-0095). The rats
128 were anesthetized with a mixture of 0.3 mg/kg of medetomidine, 4.0 mg/kg of midazolam, and 5.0
129 mg/kg of butorphanol. After partially shaving the right tibial region, a round cavity defect 1.5-2.0 mm
130 in diameter was created in the arterial center of the tibia. The site was irrigated with sterilized

131 physiological saline during drilling to prevent overheating. With the defect completed, an HAp/Col
132 cylinder was placed into the defect such that the top and bottom planes of the cylinder and the collagen
133 fiber layers were parallel to the periosteal surface of the tibia (Fig. 1). The defects were covered with
134 the tibial skin and an instant adhesive (Aron Alpha[®], TOAGOSEI, Co. Ltd., Japan).

135

136 *2.3. Specimen preparation*

137 At 3, 5, 7, 14, and 28 days after the HAp/Col grafting ($N = 6$ for each), the rats were anesthetized and
138 perfused with 4% paraformaldehyde diluted in 0.1 M phosphate buffer (pH 7.4) through the left cardiac
139 ventricle, and the tibiae were extracted and immediately immersed in the same solution for 24 h at 4°C.
140 Micro-CT images were taken, and the specimens were decalcified with 10% EDTA-2Na for 2 months.
141 The decalcified specimens were dehydrated in a series of ethanol solutions with increasing
142 concentrations, soaked in xylene, and finally embedded in paraffin. Then, 5 µm-thick sagittal paraffin
143 sections were cut to examine two regions of the HAp/Col cylinder: the lateral region facing the cortical
144 bone (perpendicular to the HAp/Col layers), and the bottom region near the marrow (parallel to the
145 collagen layers) as previously described [11,12].

146

147 *2.4. Micro-CT analysis*

148 Micro-CT images were obtained from the grafted areas using a micro-CT scanner (tube voltage 90 kV,
149 CosmoScan FX, Rigaku Corporation, Japan) following the methods in Morimoto et al. [12]. The CT
150 analyzer software CosmoScan Viewer (Rigaku Corporation) was employed for image reconstruction
151 per the guidelines described by Bouxsein et al. [13].

152

153 *2.5. Histochemistry for PHOSPHO1 and tartrate-resistant acid phosphatase*

154 After inhibition of endogenous peroxidase activity with methanol containing 0.3% hydrogen
155 peroxidase for 30 min, dewaxed paraffin sections were pretreated with 1% bovine serum albumin
156 (BSA; Serologicals Proteins Inc., Kankakee, IL) in PBS (1% BSA-PBS) for 30 min. The sections were
157 then incubated with rabbit polyclonal anti-PHOSPHO1 antibody
158 (phosphoethanolamine/phosphocholine phosphatase 1; Q8TCT1, Cusabio Technology Llc., TX)
159 diluted at 1:100 in 1% BSA-PBS at room temperature (RT) for 2 h and then incubated with horseradish
160 peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (P0399, DakoCytomation, Denmark) at a
161 dilution of 1:100 for 1 h at RT. Detection of tartrate-resistant acid phosphatase (TRAP) was conducted
162 as previously described [14].

163

164 *2.6. Regions of interest and TRAP-positive osteoclast and PHOSPHO1-reactive osteoblast*

165 *enumeration*

166 Two 300 × 900 μm rectangular regions of interest (ROIs), one located in the center of the distal side
167 of the HAp/Col cylinder and the other in the center of the bottom (marrow) side, were placed in images
168 of the sagittal sections of tibiae to represent regions where the collagen layers of the cylinder run
169 perpendicular and parallel to the bone structure, respectively (Fig. 1).

170 The total numbers of TRAP-positive osteoclasts and PHOSPHO1-reactive osteoblasts attached
171 to the HAp/Col surface and newly formed bone in each ROI were counted as described recently in
172 Haraguchi-Kitakamae et al. [15].

173

174 2.7. Statistical analysis

175 All values are expressed as means ± standard errors. Student's *t*-tests were used for two-group
176 comparisons, and one-way ANOVAs followed by Dunnett's tests were used for multiple comparisons.
177 *P*-values < 0.05 were considered to infer statistical significance.

178

179

180 3. Results

181

182 3.1. Micro-CT imaging

183 Micro-CT images from day 3 to 28 post-grafting showed the HAp/Col cylinder's upper surfaces
184 parallel to surrounding cortical bone (Fig. 2). A semi-transparent tissue formed around the cylinders
185 by day 5 (Fig. 2B), thickening into new bone by day 7 (Fig. 2C). By day 14, radiopaque trabecular
186 bone was visible around the cylinder, the surface of which had begun to disintegrate (Fig. 2D). On day
187 28, the cylinder appeared osseointegrated with adjacent cortical bone, while the bottom regions of the
188 HAp/Col cylinder showed resorption (Fig. 2E).

189

190 3.2. Chronological observations of bone development after HAp/Col cylinder grafting

191 On day 3 post-implantation, the HAp/Col cylinders showed a smooth surface surrounded by bone
192 marrow tissue (Fig. 3A). The lateral region exhibited a small amount of cellular invasion into the
193 cylinder (Fig. 3A and B). By day 5, fine trabecular bone had formed around the cylinder, with a thick
194 cell layer on the lateral surface. Immature trabeculae were observed at a distance from the lateral
195 surface, while the bottom surface showed continuous trabecular network extension (Fig. 3D–F). On
196 day 7, the cylinder had become encompassed by newly formed bone with a typical trabecular structure.
197 In the lateral region, trabeculae formation remained at a distance from the cylinder, separated by
198 fibrous stromal tissue. The bottom surface was covered with a thin bone matrix layer, from which

199 trabeculae extended outward (Fig. 3G–I). On day 14, the cylinder surface appeared irregular and
200 uneven. The lateral region lacked continuity between the new bone and the cylinder, with numerous
201 multinucleated giant cells on the surface. Rows of cells had invaded the cylinder's interior in a parallel
202 arrangement. The bottom region showed fragmentation into islands, with new bone filling the spaces
203 between them (Fig. 3J–L). By day 28, the shape of the HAp/Col cylinder had collapsed, with new bone
204 penetrating its interior. The lateral region showed integration between the HAp/Col matrix and new
205 bone tissues, while the bottom region exhibited active bone resorption with accumulated
206 multinucleated giant cells (Fig. 3M–O).

207

208 *3.3. Chronological observations of the TRAP-positive osteoclast distributions*

209 On day 3, numerous TRAP-positive osteoclasts were observed around the HAp/Col cylinder, with
210 fewer at the bottom region than at the lateral (Fig. 4A–C). By day 5, osteoclasts had become more
211 localized on the lateral surface, with fewer at the bottom (Fig. 4D–F). On day 7, many osteoclasts were
212 found on the lateral surface, and some had invaded the cylinder's interior, while the bottom region still
213 had fewer osteoclasts (Fig. 4G–I). By day 14, a significant number of TRAP-positive osteoclasts were
214 present on the cylinder's lateral surface and the surfaces of nearby newly formed bone tissue, while
215 the bottom region showed osteoclasts on new trabeculae (Fig. 4J–L). By day 28, osteoclasts had fully
216 invaded the cylinder, indicating strong resorption activity, on both lateral and bottom surfaces (Fig.
217 4M–O).

218

219 *3.4. Numbers of TRAP-positive osteoclasts*

220 We analyzed the numbers of osteoclasts attached to the HAp/Col material and newly formed bone, as
221 well as the total number of osteoclasts in the ROIs (Fig. 5). On day 3, few TRAP-positive osteoclasts
222 were observed around the cylinder, with no regional differences. From days 5 to 14, osteoclast numbers
223 increased significantly, especially in the lateral region where collagen fibers intersect. The bottom
224 region also saw an increase, but it was less pronounced. By day 28, osteoclast numbers attached to the
225 cylinder and new bone had decreased in the lateral region to levels similar to those of the bottom region.
226 However, the total osteoclast count remained higher in the lateral region. Taken together, these findings
227 suggest that osteoclasts predominantly accumulate in the lateral region of the graft, where collagen
228 fibers intersect, rather than in the bottom region, where fibers run parallel.

229

230 *3.5. Chronological observations of the PHOSPHO1-positive osteoblast distributions*

231 PHOSPHO1 is an enzyme that hydrolyzes phosphocholine and phosphoethanolamine to release
232 phosphate ions within matrix vesicles derived from osteoblasts and chondrocytes [16, 17]. Since

233 chondrocytes were not found in bone regeneration sites, we employed PHOSPHO1 as a marker of
234 bone-mineralizing osteoblasts. Three days post-surgery, no PHOSPHO1-positive osteoblasts were
235 visible on any HAp/Col cylinder surface (Fig. 6A–C). On days 5–7, osteoblasts appeared in trabecular
236 bone tissue around the cylinder (Fig. 6D and G). In lateral regions, they were seen in trabecular bone
237 adjacent to the cylinder surface, but not on it (Fig. 6E and H). However, they were detected in new
238 bone extending from the bottom surface (Fig. 6F and I). On day 14, PHOSPHO1-positive osteoblasts
239 remained in trabecular bone distant from the lateral surface (Fig. 6J and K), while lines of them were
240 noted on new trabecular bone in the bottom region (Fig. 6L). By day 28, rows of PHOSPHO1-positive
241 osteoblasts extended throughout the cylinder (Fig. 6M and N) but showed a decreasing trend in the
242 trabecular bone at the bottom region (Fig. 6O).

243 *3.6. Numbers of PHOSPHO1-positive osteoblasts*

244 We counted the PHOSPHO1-positive osteoblasts attached to the HAp/Col cylinder and the newly
245 formed bone in the ROIs of the lateral and bottom regions (Fig. 7). During days 5–14, PHOSPHO1-
246 positive osteoblasts predominantly increased in the bottom region, with significantly greater numbers
247 counted in the bottom region than in the lateral region throughout this period. However, by the 28th
248 day, there was no significant difference in the numbers of PHOSPHO1-positive osteoblasts between
249 the two regions.

250

251

252 **4. Discussion**

253

254 We aimed to clarify whether the orientation of collagen fibers in a bone substitute material composed
255 of HAp/Col fibers affects bone resorption and bone formation. To achieve this, we implanted an
256 HAp/Col graft composed of layered collagen plates into the diaphysis of rat tibiae and conducted
257 histological analyses. Our findings revealed distinct patterns of osteoclast and osteoblast localization
258 in different regions of the implant during the early post-implantation period (5–14 days). In the lateral
259 region, where collagen fiber ends were exposed, numerous osteoclasts accumulated on the HAp/Col
260 cylinder surface and resorbed them, with minimal osteoblasts present. Therefore, bone remodeling may
261 occur in the lateral region. Conversely, the bottom region, where collagen fibers ran parallel, exhibited
262 predominantly osteoblastic bone formation, with lower osteoclast accumulation, suggesting bone
263 modeling (Fig. 8). These findings suggest that the orientation of collagen fibers influences the early
264 localization of osteoclasts and osteoblasts, uncoupling osteoclastic bone resorption and osteoblastic
265 bone formation, which affects bone modeling and has significant implications for bone regeneration.

266 Hydroxyapatite and beta-tricalcium phosphates are well-known osteoconductive materials that
267 serve as scaffolds for bone cell migration and attachment [18–23]. Previous research indicates that
268 bone substitute material replacement is similar to bone remodeling, with osteoclasts first resorbing the
269 osteoconductive material, followed by osteoblast migration and bone matrix addition [23,24], making
270 it distinct from bone modeling, even during bone regeneration.

271 To facilitate cell infiltration into calcium phosphate-based bone substitutes, various
272 improvements have been made, including optimizing granule and pore sizes and incorporating
273 collagen fibers [25–28]. However, the orientation of collagen fibers has received relatively little
274 attention. Therefore, we examined how collagen fiber orientation affected bone resorption and
275 formation. To do this, it is necessary to exclude other factors that would induce bone resorption and
276 bone formation, for instance, mechanical stress and the osteocytic lacunar canalicular system [30–32].
277 We embedded the HAp/Col graft avoiding tight contact between the embedded cylinders and the
278 surrounding bone tissue of the rat tibiae, avoiding mechanical stress. In addition, there was no living
279 osteocytic network in the HAp/Col cylinder. Therefore, we believe that our study excluded these
280 factors. We hypothesize that, in the absence of mechanical stress, the orientation of collagen fibers
281 affects the localization of osteoclast and osteoblast accumulation.

282 One may wonder “Why did osteoclasts accumulate in the graft’s lateral region, where the fiber
283 ends are exposed at the surface, while bone formation by osteoblasts was induced in the bottom region,
284 where the collagen fibers run parallel to the surface?” According to the studies by Hasegawa et al. [33],
285 osteoblast projections show two types: one that extends perpendicularly to the bone surface to connect
286 with osteocytes and another that encompasses collagen bundles. Therefore, there is a relationship
287 between the orientation of osteoblast projections and that of collagen fiber bundles, and the present
288 study’s findings suggest that HAp/Col fibers running parallel to the secretion surface of osteoblasts
289 may be more advantageous, *e.g.*, for cell adhesion via integrins [34]. On the other hand, the bone
290 resorption compartment of osteoclasts, which is also responsible for collagen reabsorption, has a
291 characteristic ruffled border, and it seems that collagen fibers oriented perpendicularly to this
292 resorption surface may be more easily absorbed. In addition, several researchers have mentioned that
293 HAp crystals have different properties depending on their surface orientation. Specifically, the *m*-
294 surface and *c*-surface of HAp exhibit differences in crystal solubility, protein adsorption, and cell
295 growth [35,36]. Aizawa et al. [37] demonstrated that osteoblasts promoted mineralization when
296 cultured *in vitro* on HAp ceramics with the *m*-surfaces exposed. In our study, the bottom surface of the
297 HAp/Col cylinders, where the *m*-surfaces of the HAp crystals is exposed, induced osteoblastic bone
298 formation. Thus, the orientation of both collagen fibers and HAp crystals may contribute to osteoblast
299 induction.

300 The distinct osteoclast and osteoblast accumulation patterns associated with the different
301 HAp/Col fiber orientations were most pronounced during the early stages of graft recovery in this
302 study. As bone resorption and bone formation progressed, the distribution of osteoclasts and osteoblasts
303 within the HAp/Col cylinder became indistinct over time. This is likely due simply to the nature of the
304 bone remodeling process, which has previously been described [38,39]. Therefore, in the context of
305 bone reconstruction, if the orientation of collagen fibers in the HAp/Col graft can be standardized to
306 induce osteoclast or osteoblast accumulation in desired areas, it may be possible to guide bone
307 regeneration in a controlled manner during the early stages.

308 One limitation of this study is that, while the HAp/Col fibers were aligned in one plane, they still
309 pointed in various directions within that plane, meaning that the structure did not fully mimic the
310 geometric structure of lamellar bone tissue. However, given this study's results, HAp/Col graft designs
311 with refined collagen fiber orientations are anticipated. Additionally, while enumerating TRAP-
312 positive osteoclasts and PHOSPHO1-positive osteoblasts, especially in the late stages, it was hard to
313 distinguish osteoclasts and osteoblasts located on the HAp/Col surface from those on the newly formed
314 bone. Therefore, in this study, we counted the cells located on the cylinder and the new bone together.
315 Additionally, further analysis is required to elucidate the mechanisms by which collagen fiber and HAp
316 crystal alignment induce distinct localizations of osteoclasts and osteoblasts.

317

318

319 **5. Conclusions**

320

321 This study's results suggest that when an HAp/Col graft with HAp/Col fibers in a planar orientation is
322 implanted into the tibial cavity of rats, osteoclasts accumulate in the region where the collagen fiber
323 ends are exposed, while osteoblasts tend to adhere and induce bone formation in the regions where the
324 collagen fibers run parallel to the bone tissue's surface. In bone reconstruction, controlling the
325 orientation of collagen fibers in HAp/Col bone substitute materials may allow for targeted bone
326 resorption or the promotion of bone formation in specific regions, potentially enabling surgeons to
327 guide bone regeneration in a predictable manner.

328

329

330 **Ethical standards**

331 All animal experiments were performed in accordance with the Japanese Act on Welfare and
332 Management of Animals. The protocol was approved by the Institutional Animal Care and Use

333 Committee of Hokkaido University (approved research proposal #23-0095).

334

335 **Competing interests**

336 The authors declare no competing interests.

337

338 **Contributors**

339 Hasegawa T and Kikuchi M designed this study and prepared the final draft of the article. Sakakibara
340 M is the first author in charge of writing the original draft and analyzing and interpreting the data.
341 Haraguchi-Kitakamae M, Shi Y, Li W, Liu X, and Hongo H contributed to the histochemical analysis.
342 Cui J and Yamamoto T worked on the statistical analyses and critically read the manuscript. Sato Y
343 and Amizuka N participated in the discussion, editing, and formatting of the manuscript.

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345

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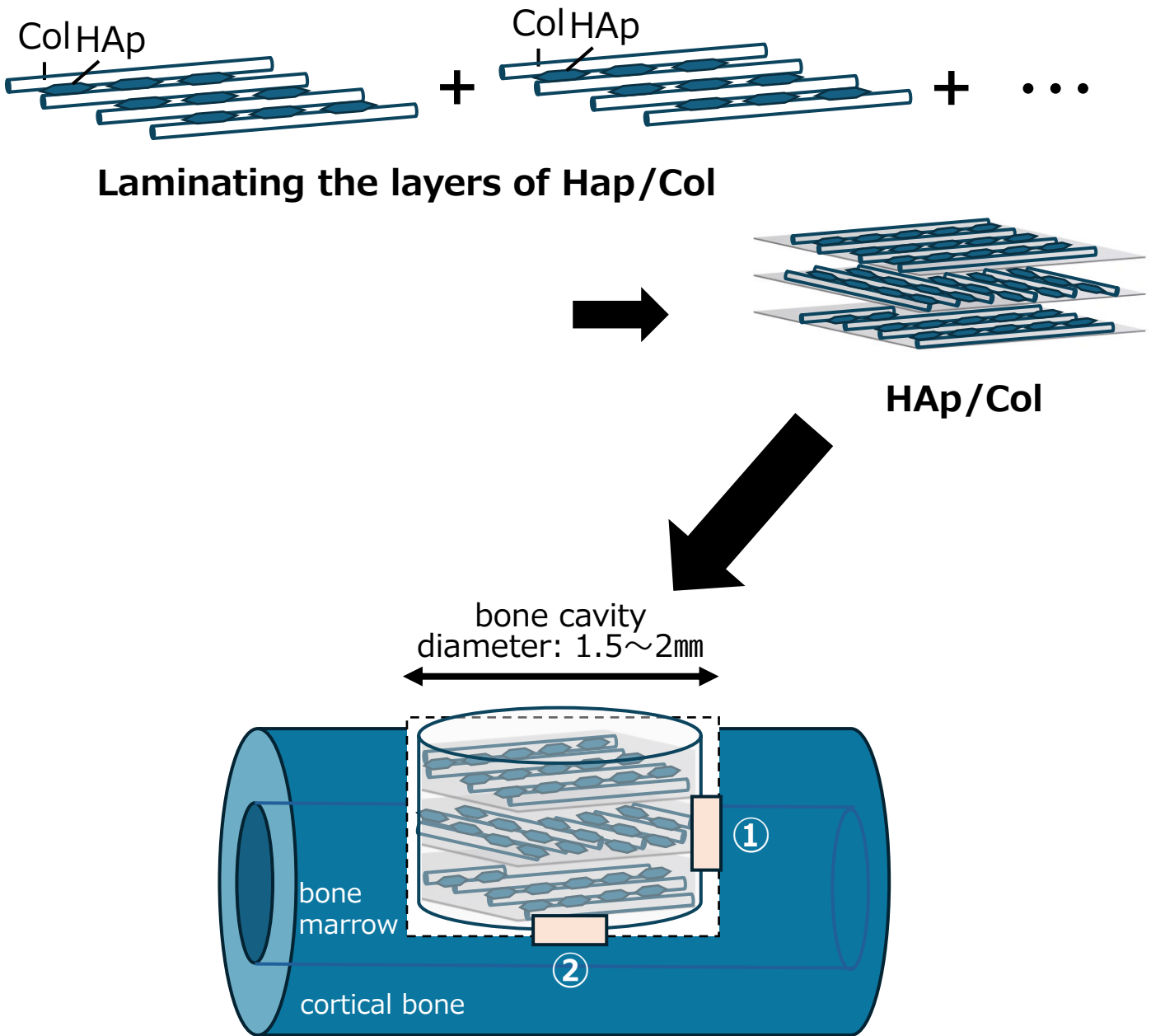
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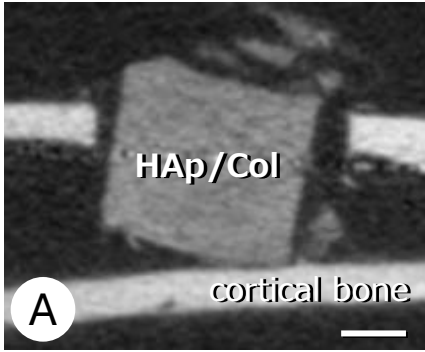


Regions of Interest

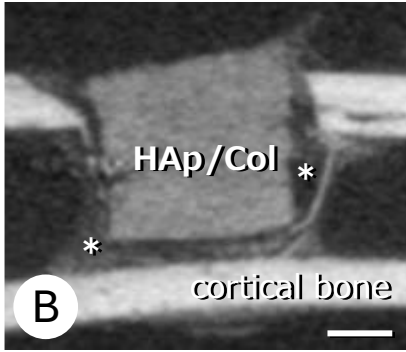
- ① : the region perpendicular to the collagen layers: lateral region
- ② : the region parallel to the collagen layers: bottom region

Figure 1

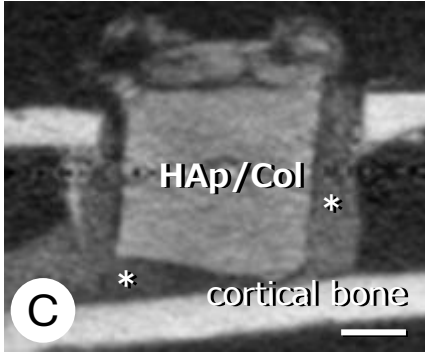
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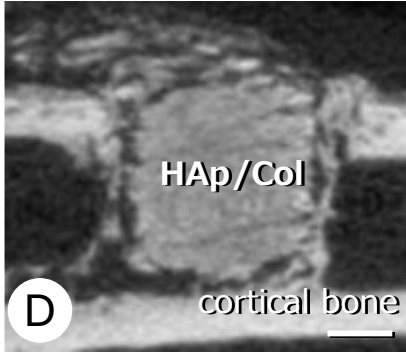
Day 5



Day 7



Day 14



Day 28

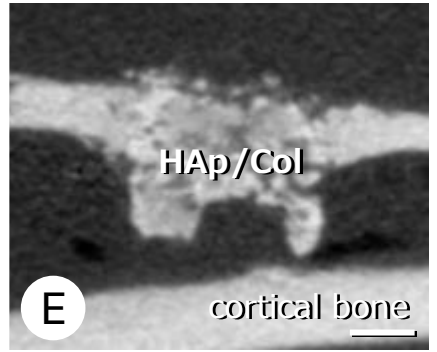


Figure 2

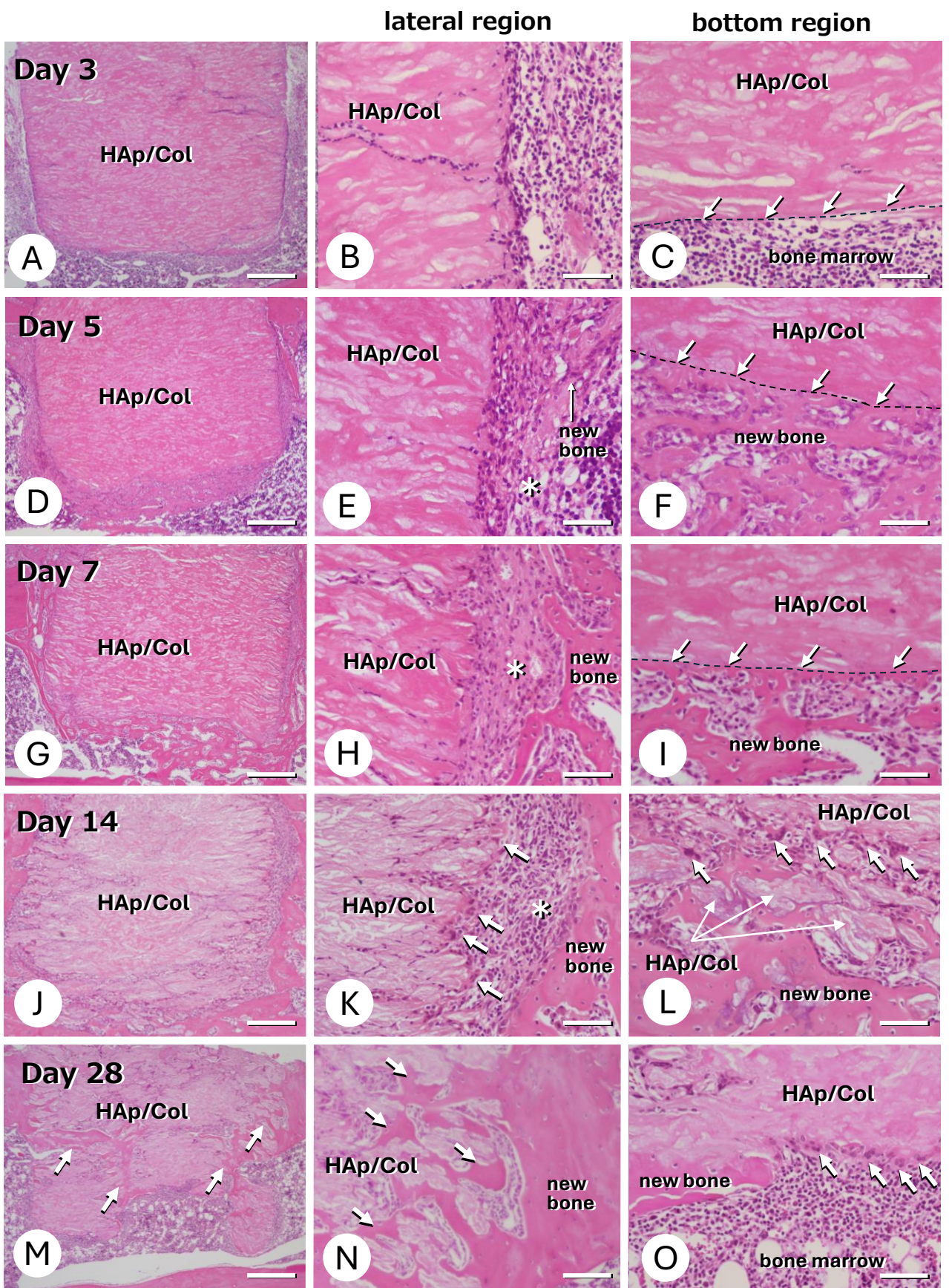


Figure 3

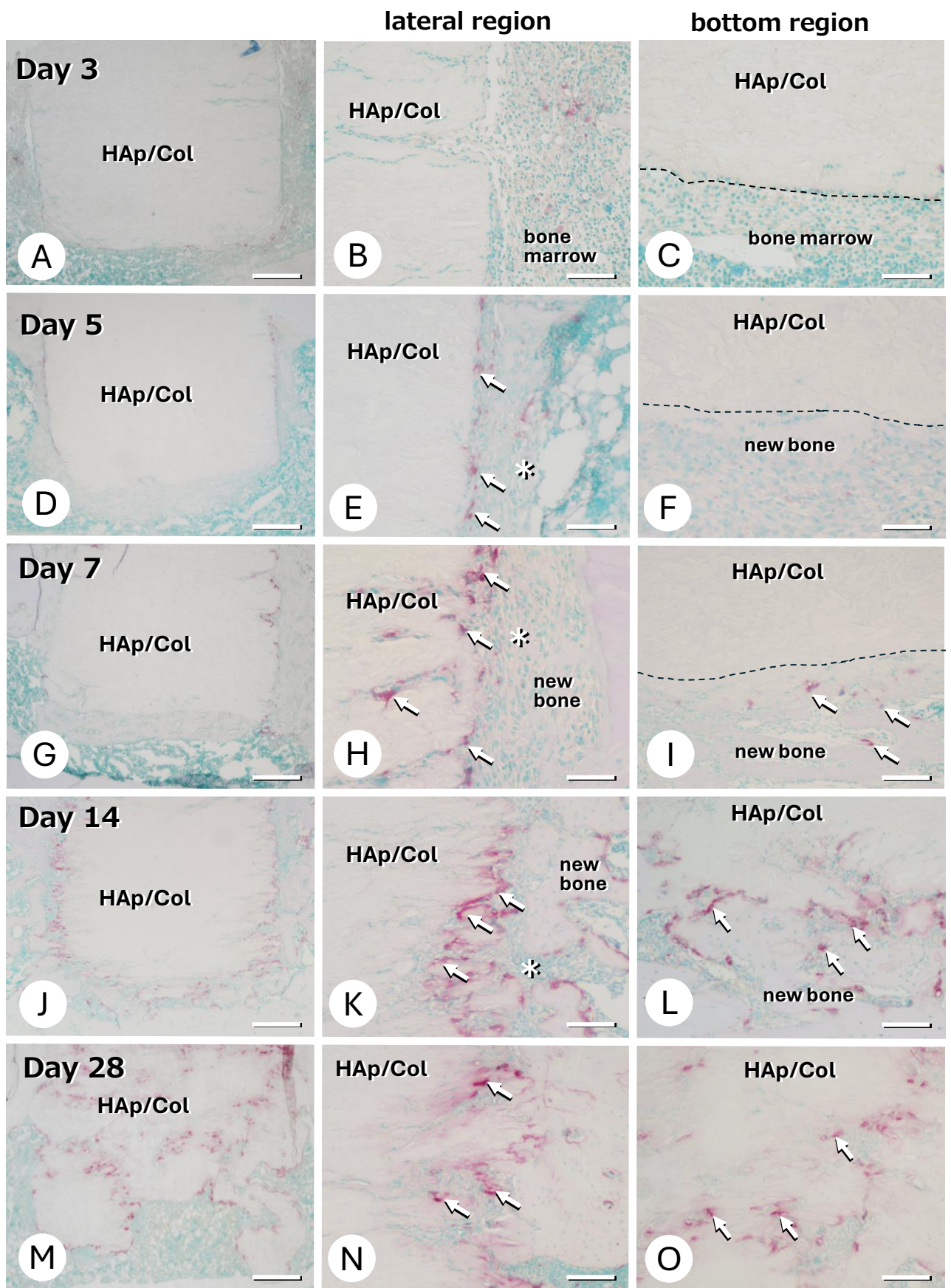
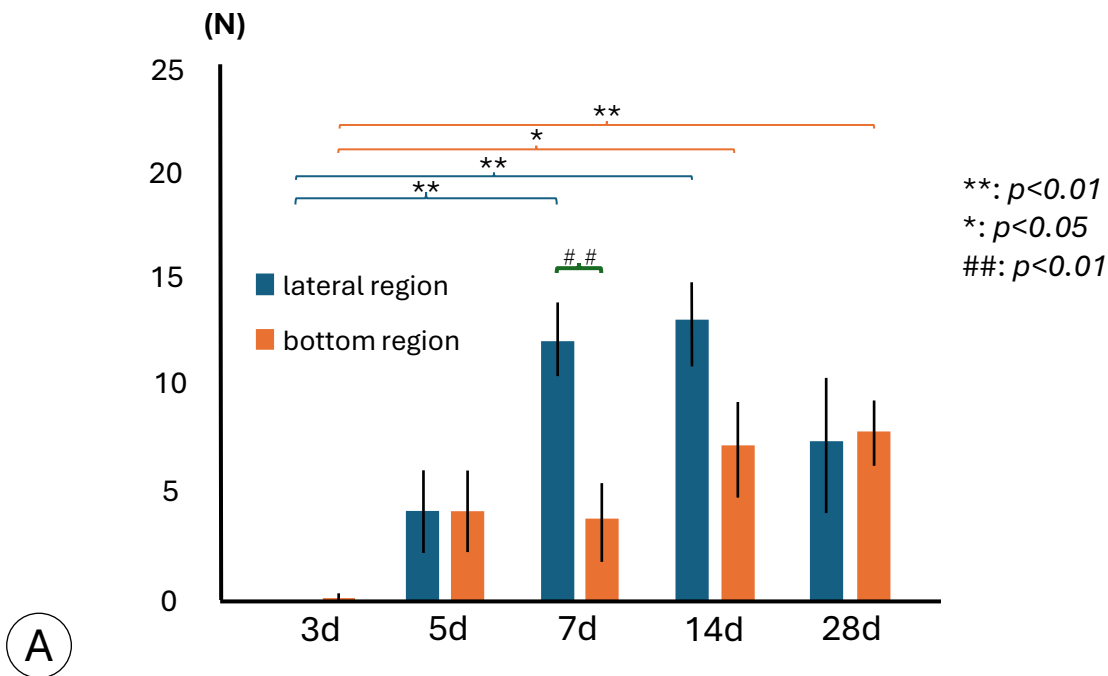


Figure 4

The numbers of TRAP-positive osteoclasts on the HAp/Col and newly-formed bone in ROIs



The total numbers of TRAP-positive osteoclasts in ROIs

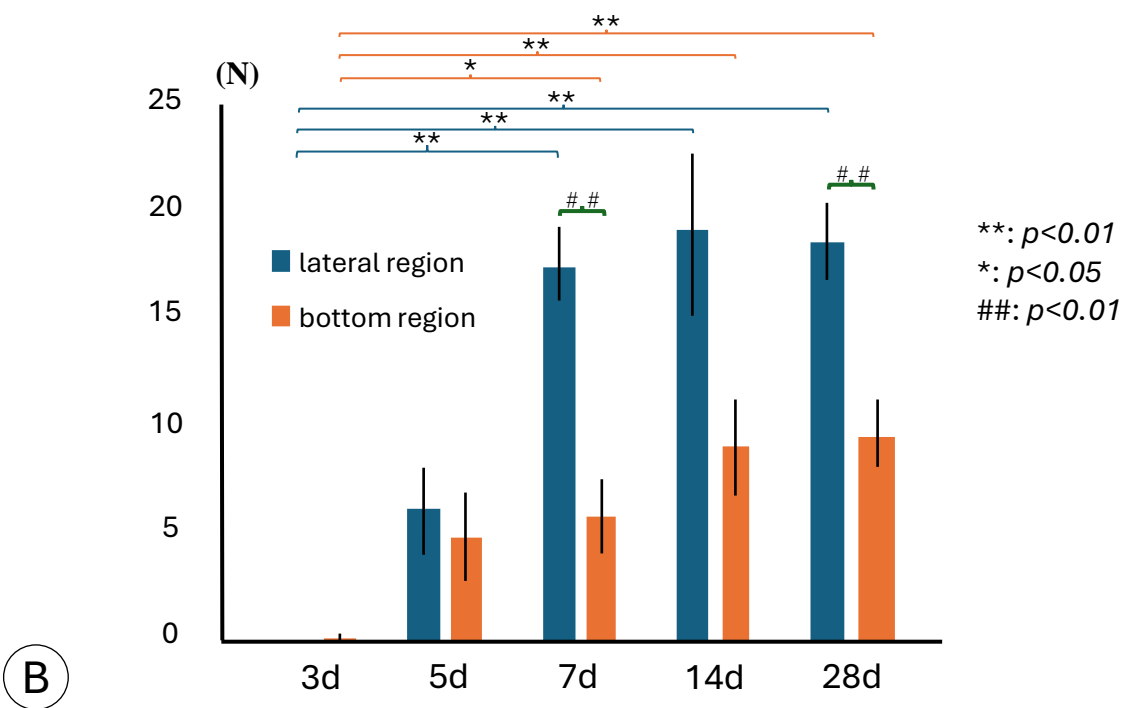


Figure 5

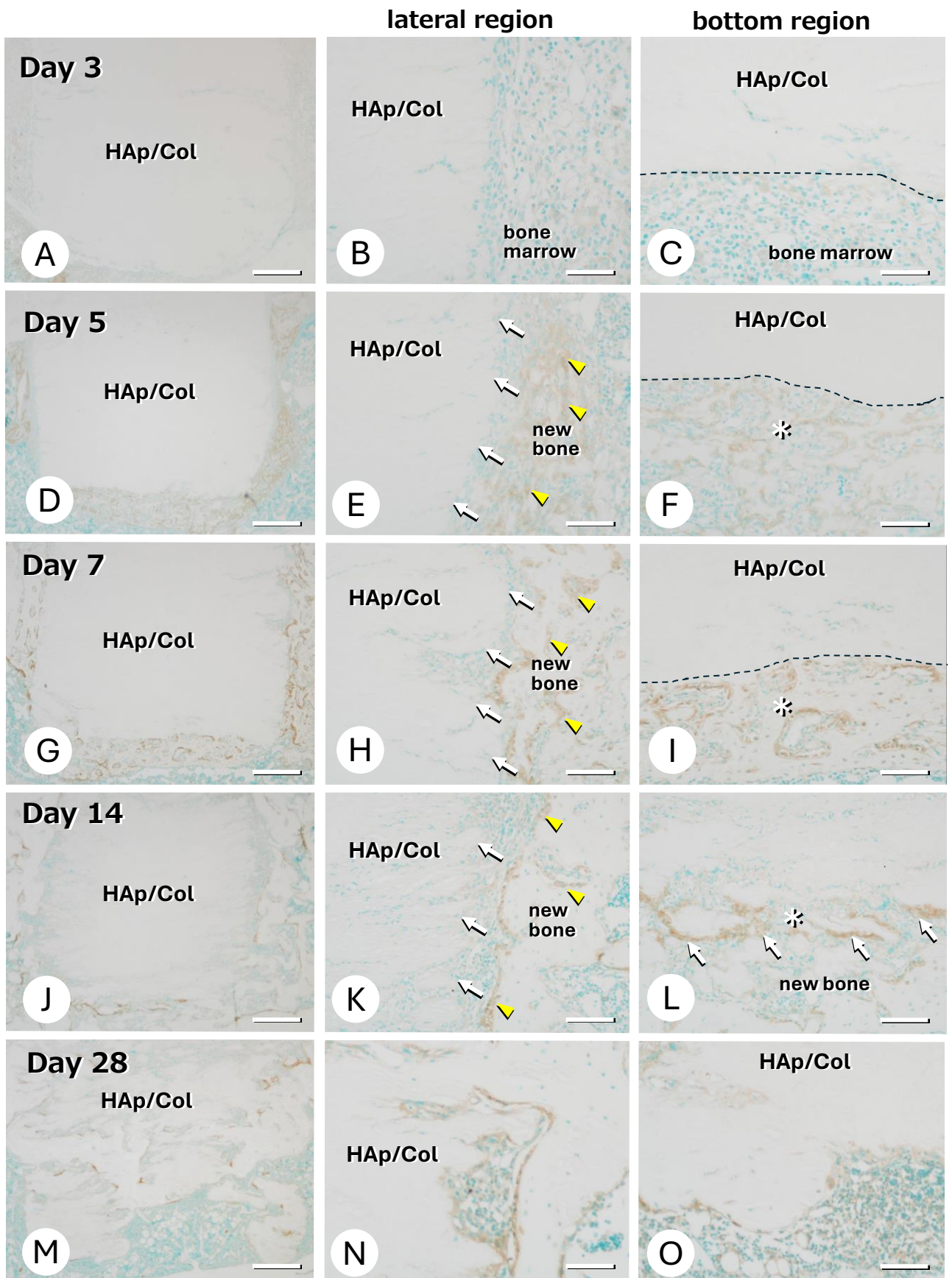


Figure 6

The numbers of PHOSPHO1-positive osteoblasts on the HAp/Col and newly-formed bone in ROIs

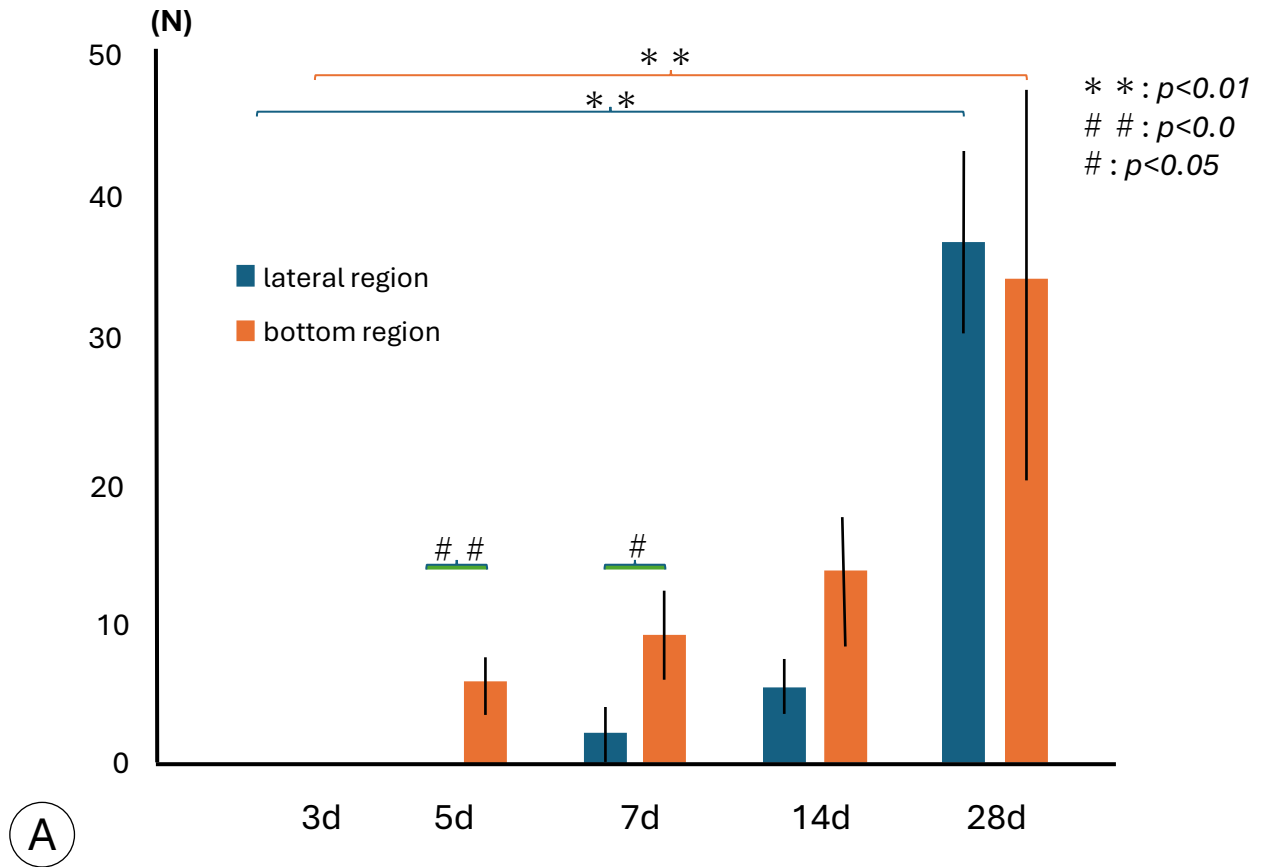
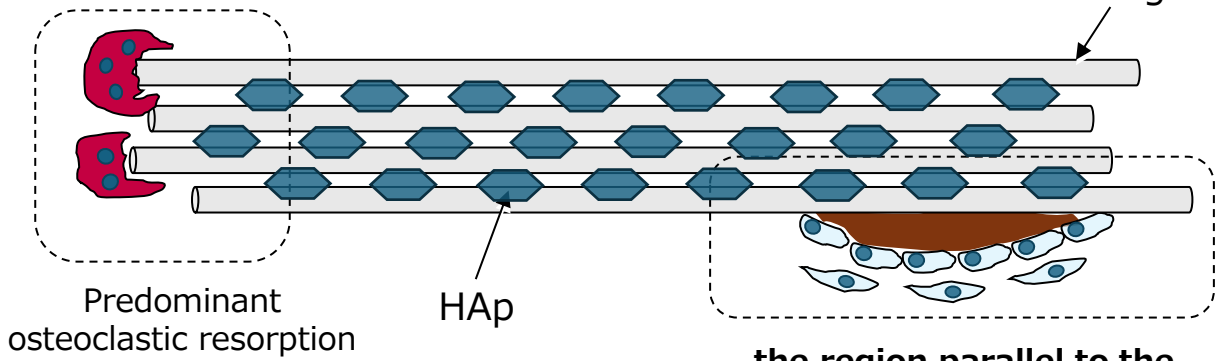


Figure 7

the region perpendicular to the collagen layers: lateral region



the region parallel to the collagen layers: bottom region

Figure 8

501 **Figure legends**

502

503 **Figure 1**

504 *Schematic design of HAp/Col embedding into rat tibiae*

505 A cylinder of HAp/Col with a diameter of 1.5 mm and a height of 1.5 mm was implanted into a 1.5–
506 2.0 mm diameter bone defect in the arterial center of the right tibia of 10-week-old male Wistar rat.
507 Two 300 × 900 μm rectangular regions of interest (ROIs) were set up located in the center of the lateral
508 (perpendicular to the collagen layers) and bottom (parallel to the collagen layers) regions of the
509 HAp/Col cylinder.

510

511 **Figure 2**

512 *Micro-CT images of the HAP/Col cylinder taken 3–28 days after grafting into the bone cavities*

513 The micro-CT images reveal a semi-transparent tissue around the HAp/Col cylinders on days 5 and 7
514 that was not present 3 days after grafting (asterisks, **A-C**). By day 14, trabecular bone is apparent
515 around the cylinder, while the surface appears roughened (**D**). On day 28, the HAp/Col cylinder and
516 the adjacent cortical bone are well-integrated with each other, and the bottom regions of the cylinder
517 have been eroded (**E**). The scale bar represent 500 μm.

518

519 **Figure 3**

520 *H-E staining on HAp/Col cylinder graft*

521 Panels **A-C**, **D-F**, **G-I**, **J-L**, and **M-O** were obtained 3, 5, 7, 14, and 28 days after the HAp/Col
522 grafting, respectively. Panels **A**, **D**, **G**, **J**, and **M** are low-magnification images of the HAP/Col cylinder.
523 Panels **B**, **E**, **H**, **K**, and **N** and **C**, **F**, **I**, **L**, and **O** are histological images from the lateral and bottom
524 regions, respectively. On day 3, the surface of the grafted HAp/Col cylinders look smooth, being
525 surrounded by bone marrow tissue (**A-C**). The dotted line indicates the boundary between the HAp/Col
526 graft and the bone marrow (**C**). On days 5 and 7, trabecular new bone is seen around the HAp/Col
527 cylinder (**D**, **G**). Trabecular new bones are continuous with the bottom surface of the cylinder (**F**, **I**),
528 whereas the new bones are discontinuous from the cylinder's lateral surface, with intervening stromal
529 tissues (asterisks, **E**, **H**). The dotted line indicates the boundary between the HAp/Col graft and the
530 newly formed bone (**F**, **I**). On day 14, in the lateral region, no continuity between the newly formed
531 bone and the HAp/Col cylinder is seen yet due to intervening stromal tissue (asterisks, **J**, **K**). Numerous
532 multinucleated cells are localized on the lateral surface of the HAp/Col cylinder (arrows, **K**), while the

533 bottom region has been fragmented into islands, with newly formed bone filling the spaces between
534 them (L). Note the multinucleated giant cells at the boundary between the HAp/Col graft and the new
535 bone (arrows, L). By day 28, the HAp/Col cylinder had collapsed, and newly formed bone had
536 penetrated its interior in both the lateral and bottom regions (M–O). The scale bars in A, D, G, J, and
537 M represent 300 μm , while those in B, C, E, F, H, I, K, L, N, and O represent 100 μm .

538

539 **Figure 4**

540 *Distribution of TRAP-positive osteoclasts*

541 Panels A–C, D–F, G–I, J–L, and M–O were obtained 3, 5, 7, 14, and 28 days after the HAp/Col
542 grafting respectively. Panels A, D, G, J, and M are low-magnification images of the HAp/Col cylinders.
543 Panels B, E, H, K, and N and C, F, I, L, and O are histological images from the lateral and bottom
544 regions, respectively. On day 3, TRAP-positive osteoclasts are nearly absent around the HAp/Col
545 cylinder (A–C). The dotted line indicates the boundary between the HAp/Col graft and the bone
546 marrow (C). On day 5, several TRAP-positive osteoclasts (red color, arrows) are localized along the
547 lateral surface of the cylinder but not the bottom surface (D–F). The dotted line indicates the boundary
548 between the HAp/Col and newly formed bone (F). The asterisks indicates stromal tissues (E, H, K).
549 By days 7 and 14, many TRAP-positive osteoclasts (arrows) have accumulated on the bumpy lateral
550 surface of the HAp/Col cylinder (G, H, J, K), allowing them to invade the cylinder’s interior (H, K).
551 In contrast, in the bottom region, TRAP-positive osteoclasts (arrows) are seen in small numbers on day
552 7, but many were present on day 14 (I, L). The dotted line indicates the boundary between the HAp/Col
553 graft and the newly formed bone (I). By day 28, TRAP-positive osteoclasts had completely invaded
554 the HAp/Col cylinder (M–O). The scale bars in A, D, G, J, and M represent 300 μm , while those in
555 B, C, E, F, H, I, K, L, N, and O represent 100 μm .

556

557 **Figure 5**

558 *Statistical analysis of the distribution of TRAP-positive osteoclasts*

559 Panel A shows the number of TRAP-positive osteoclasts attached to the HAp/Col cylinder’s surfaces,
560 while panel B represents the total number of osteoclasts in the ROIs. The data are shown as the mean
561 \pm SE. Significant differences ($P < 0.05$) among means are represented using symbols: *, $P < 0.01$ and
562 **, $P < 0.001$, for Dunnett’s test results, and ^{###}, $P < 0.001$ for student’s *t*-test results.

563

564 **Figure 6**

565 *Distribution of PHOSPHO1-positive osteoblasts*

566 Panels **A–C**, **D–F**, **G–I**, **J–L**, and **M–O** were obtained from 3, 5, 7, 14, and 28 days after the HAp/Col
567 grafting, respectively. Panels **A**, **D**, **G**, **J**, and **M** are low-magnification images of the HAp/Col
568 cylinders. Panels **B**, **E**, **H**, **K**, and **N** and **C**, **F**, **I**, **L**, and **O** are histological images from the lateral and
569 bottom regions, respectively. On day 3, PHOSPHO1-positive osteoblasts are nearly absent from the
570 surface of the HAp/Col cylinder (**A–C**). The dotted line indicates the boundary between the HAp/Col
571 graft and the bone marrow (**C**). On days 5–7, PHOSPHO1-positive osteoblasts (brown color) can be
572 seen in the new bone tissue around the HAp/Col cylinder (**D**, **G**). In the lateral regions of the HAp/Col
573 cylinder, PHOSPHO1-positive osteoblasts (arrowheads) were seen in the new bone tissue at a distance
574 from the lateral cylinder surface but not on the surface itself (**E**, **H**; note that no PHOSPHO1-positive
575 cells are visible on the HAp/Col cylinder surface, as indicated by arrows). However, PHOSPHO1-
576 positive osteoblasts are located in the new bone tissue (asterisks) extending from the bottom surface
577 of the cylinder (**F**, **I**). The dotted line indicates the boundary between the HAp/Col graft and the newly
578 formed bone (**F**, **I**). On day 14, PHOSPHO1-positive osteoblasts (arrowheads) can be seen on the
579 trabecular bone at a distance from the lateral surface (arrows) of the HAp/Col cylinder but are still
580 absent on the surface (**K**), whereas lines of PHOSPHO1-positive osteoblasts (arrows) were noted on
581 the newly formed bone (asterisk) in the bottom region (**L**). By day 28, rows of PHOSPHO1-positive
582 osteoblasts are observed throughout the HAp/Col cylinder (**M–O**). The scale bars in **A**, **D**, **G**, **J**, and
583 **M** represent 300 μm , while those in **B**, **C**, **E**, **F**, **H**, **I**, **K**, **L**, **N**, and **O** represent 100 μm .

584

585 **Figure 7**

586 *Statistical analysis of the distribution of PHOSPHO1-positive osteoblasts*

587 Panel **A** shows the number of TRAP-positive osteoclasts attached to the HAp/Col cylinder's surface,
588 while panel **B** represents the total number of osteoclasts in the ROIs. The data are shown as the mean
589 \pm SE. Significant differences ($P < 0.05$) among means are represented using symbols: *, $P < 0.01$ and
590 **, $P < 0.001$, for Dunnett's test results, and ^{##}, $P < 0.01$ and [#], $P < 0.05$ for student's *t*-test results.

591

592 **Figure 8**

593 *A schematic illustrating the findings of our study*

594 After embedding an HAp/Col graft with laminated plates of horizontally orientated collagen fibers into
595 the cavity defect in the tibia of a rat, osteoclasts accumulate in the region where the collagen fiber ends
596 are exposed, while osteoblasts tend to adhere and induce bone formation in the regions where the
597 collagen fibers run parallel to the bone tissue surface.

598

599