Hyaluronan stimulates chondrogenic gene expression in human meniscus cells

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Hyaluronan stimulates chondrogenic gene expression in human meniscus cells

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Hiroto Inoue: Experiments, cell cultures. Yuya Kodama: Data analysis.

Toshifumi Ozaki: Laboratory organization.

ABSTRACT

Purpose/Aim of the Study: Inner meniscus cells have a chondrocytic phenotype, whereas outer meniscus cells have a fibroblastic phenotype. In this study, we examined the effect of hyaluronan on chondrocytic gene expression in human meniscus cells.

Materials and Methods: Human meniscus cells were prepared from macroscopically intact lateral meniscus. Inner and outer meniscus cells were obtained from the inner and outer halves of the meniscus. The cells were stimulated with hyaluronan diluted in Dulbecco's modified Eagle's medium without serum to the desired concentration (0, 10, 100, and 1000 μg/mL) for 2–7 days. Cellular proliferation, migration, and polymerase chain reaction analyses were performed for the inner and outer cells separately. Meniscal samples perforated by a 2-mm-diameter punch were maintained for 3 weeks in hyaluronan-supplemented medium and evaluated by histological analyses.

Results: Hyaluronan increased the proliferation and migration of both meniscus cell types. Moreover, cellular counts at the surface of both meniscal tissue perforations were increased by hyaluronan treatments. In addition, hyaluronan stimulated α1(II) collagen expression in inner meniscus cells. Accumulation of type II collagen at the perforated surface of both meniscal samples was induced by hyaluronan treatment. Hyaluronan did not induce type I collagen accumulation around the injured site of the meniscus.

Conclusion: Hyaluronan stimulated the proliferation and migration of meniscus cells. Our results suggest that hyaluronan may promote the healing potential of meniscus cells in damaged meniscal tissues.

Keywords: Meniscus, Hyaluronan, Type II collagen, Inner meniscus, Outer meniscus, Chondrocytic gene expression

Introduction

Hyaluronan (HA) is a linear macromolecular polymer composed of a repeated structure of the disaccharide N-acetyl-D-glucosamine and D-glucuronic acid.[1] The number of disaccharide repeats is over 10,000, and the molecular weight ranges from 10^3 to 10^4 kDa. HA is a component of the extracellular matrix (ECM) of various structures and plays a vital role in structural control and homeostasis, including neovascularization, branching morphogenesis, cell migration, and differentiation.[2-4] HA has a high water-retention capacity and forms an elastic network structure that HA promotes cell differentiation and migration.[5] Some HA functions are thought to derive from its physiochemical characteristics, while HA controls cell behavior at the same time by binding to specific cell surface receptors and binding proteins.[6, 7] Normal human meniscus is composed of 72% water, 22% collagen, 0%–8% glycosaminoglycan, and 0%–12% DNA.[8] In the bovine meniscus, HA occupies 4%–5% and 10% of the total glycosaminoglycan in the inner and outer regions, respectively.[9] HA is a component of proteoglycan aggregaten,[10] which has the ability to increase proteoglycan synthesis, prevent glycosaminoglycan release from the cartilage matrix, and stimulate tissue inhibitor of metalloproteinase-1 in articular chondrocytes.[11] However, most studies used animal meniscus and cells, and the effects of HA on human meniscus cells remain unclear. Thus, the effects of HA treatment on the proliferation and migratory ability of human meniscus cells were considered in this study.

HA is currently used as a treatment for gonarthrosis (knee osteoarthritis). The known effects of HA on gonarthrosis include suppression of cartilage degeneration and proteoglycan migration outside the cartilage matrix, cartilage outer layer protection,[12, 13] synovial fluid normalization,[14] improvement in range of motion, and relief of knee joint pain.[15, 16] In vitro, HA has been observed to stimulate the production of tissue inhibitor of metalloproteinase-1 in chondrocytes, inhibit neutrophil-mediated cartilage degradation, and attenuate interleukin-1-induced matrix degeneration and chondrocyte cytotoxicity.[17] Articular chondrocytes cultured in the presence of HA have a significantly greater rate of DNA proliferation and ECM production, with increased matrix deposition of chondroitin-6-sulfate and type II collagen, compared with chondrocytes cultured without HA.[18] We previously conducted a comparative study on the biological characteristics of meniscus cells. In the primary culture, the inner cells have a small triangular structure, and outer cells are long and narrow.[19] The gene expression of α1(II) collagen (COL2A1), chondromodulin-I, and SRY-type HMG box-9 in inner cells was distinctive.[19-22] In addition, lipid droplet accumulation was observed by inducing differentiation of both the inner and outer cells to the adipocyte lineage; however, increase in the production of the chondrocytic matrix was only observed in the inner cells.[19] From these findings, the cells in the parenchyma of the inner meniscus region seem to maintain chondrocyte-like characteristics in both structure and gene expression, whereas cells in the outer region have fibroblast-like

characteristics within a dense connective tissue. In animal models, intra-articular HA injections improve the healing process,[23] stimulate collagen remodeling, and inhibit meniscal swelling after partial menisectomy.[11] Because the inner cells maintain chondrocyte-like characteristics, they might respond to HA treatment similarly to articular chondrocytes.

The meniscus is a relatively avascular structure with limited peripheral blood supply, and the following three zones determined healing prognosis for meniscal lesions: red, red/white, and white zone. Blood vessels are abundant in the outer region, the “red-zone”, defined as the area beyond the inner two-thirds of the meniscus. Within the inner region, the “white zone” and “red/white zone”, no blood vessels are present or avascular, wherein poor results of the meniscus treatment have been attributed.[24] In the clinical setting, a cut in the "red zone” may heal by itself, or can often be repaired with surgery. In contrast, tears in this "white zone” cannot heal. These tears are often in thin, and worn cartilage. Histologically, the meniscus, a fibrocartilaginous tissue, contains multiple cell types, with fibrochondrocytic cells in the parenchyma of the inner meniscus and fibroblast-like cells in the outer region, reportedly leading to differing reactions to mechanical stress.[25]

Based on these findings, we hypothesized that the effects of HA may differ between injuries to the inner region and outer region of the meniscus. In the present study, we used an ex vivo organ-culture model to evaluate the effects of HA treatment on injuries to human inner and

outer meniscus cells. Furthermore, the expression change in COL2A1, which is an articular cartilaginous tissue gene, and the effects of HA treatment on injuries to the inner and outer regions were examined.

Materials and methods

Cells and cell culture: Institutional Review Board approval and informed consent were obtained before all experimental studies. Macroscopically intact lateral meniscus was obtained during total knee arthroplasty in patients suffering from medial osteoarthritis of the knee. Patients with sufficient articular cartilage of the lateral femoral condyle and undegenerated lateral meniscus on magnetic resonance imaging were selected. The selected patients were 58, 61, 63, 69, 71, and 76 years old (n = 6). Inner and outer meniscus cells were prepared from the meniscal samples.[19, 26] In brief, synovial/capsular tissues and the superficial zones of the meniscus were removed carefully. The width of the obtained lateral meniscus was 10–14 mm. The center line of the meniscal width (5–7 mm from the inner edge) was marked. Inner and outer meniscus tissues were prepared by careful cutting along the center line. Inner and outer meniscus cells were prepared by collagenase (Sigma, St. Louis, MO, USA) treatment.[19, 26] After collagenase digestion, attached cells (passage 0) were maintained with Dulbecco's modified Eagle's medium (DMEM, Wako, Osaka, Japan) containing 10% fetal bovine serum (FBS; HyClone, South Logan, UT), and 1%

penicillin/streptomycin (Sigma). Meniscus cells were used between passages 1 and 2. The cell seeding density was 20% -30% (10,000 cells/cm^2) on the dish.

HA stimulation: Sodium HA [MW, 500-1200 kDa; kindly provided by Seikagaku Kogyo (Tokyo, Japan) and Kaken Pharmaceutical (Tokyo, Japan)], was diluted in DMEM without serum to the desired concentration (0, 10, 100, and 1000 µg/mL).

Cell proliferation assay: Cells were cultured for 2–7 days in serum-free DMEM with HA (0, 10, 100, or 1000 µg/mL). Cell proliferation assays were performed as described.[20, 27, 28] Cells (5,000 cells/well) were incubated on 96-well plates for 12 h prior to the addition of the cell proliferation reagent water-soluble tetrazolium (WST)-1 (Roche Diagnostics, Basel, Switzerland). Optical density (OD) was measured at evaluation and control wavelengths of 450 and 630 nm, respectively. Data obtained by subtracting 630-nm readings from 450-nm readings were used for evaluation (5 wells each).

Cell migration assay: Chemotaxis assays were performed using a modified Boyden chamber (48-well chemotaxis chamber AP48, Neuro Probe, Gaithersburg, MD, USA).[23, 27, 28] Collagen-coated polycarbonate membrane (5 µm pore, Neuro Probe) was placed over the bottom chamber filled with DMEM containing 5% FBS and 0 to 1000 µg/mL HA. Fifty microliters of cell suspension per well (5.0×10^3 cells/well in DMEM) was seeded in the upper wells. The upper and lower wells were separated by a filter coated with type I collagen. The chambers were

incubated for 4 h at a temperature of 37 °C. At the end of the incubation, filters were removed and stained with Diff-Quik (Sysmex International Reagents Co., Hyogo, Japan). Migrated cells were counted and expressed as migrated cells per high-power field.

Quantitative real-time polymerase chain reaction (PCR) analysis: RNA samples were obtained from cultured meniscus cells. Total RNAs were isolated using Isogen reagent (Nippon Gene, Toyama, Japan). RNA samples (500 ng) were reverse-transcribed with ReverTra Ace (Toyobo, Osaka, Japan). The cDNAs underwent PCR amplification in the presence of specific primers using rTaq DNA polymerase (TaKaRa, Ohtsu, Japan). For all RT-PCR fragments, the reaction was allowed to proceed for 28–37 cycles. The following specific primer sets were used: α1 (I) and α1 (II) collagen (COL1A1 and COL2A1) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH).[19, 20] Quantitative real-time PCR analyses were performed using FastStart DNA Master SYBR Green I kit (Roche Diagnostics). The cycle number crossing the signal threshold was selected in the linear part of the amplification curve. Amplification data of G3PDH were used for normalization. Relative mRNA levels were normalized with the level of meniscus cells with no HA treatment for every sample.

Histological analysis: The lateral meniscus was cut into fragments having 1-cm width. Meniscal samples were perforated with a 2-mm diameter punch in the inner 1/4 and outer 1/4 regions to create full-thickness defects as a meniscal injury model (Supplemental Fig. A-E). Although the

circular defect of the meniscus would not occur clinically, we investigated the perforated surface of the defect in our meniscal injury model. Meniscal samples were cultured for 3 weeks in a medium containing 0 or 1000 μg/mL of HA. Meniscal surfaces of the inner and outer regions were evaluated as the superficial layer. The cell numbers for the meniscus superficial layer, perforated surface, were calculated at a length of 200 μm and compared (at 6 locations). The adjacent areas were calculated at an area of 200 × 200 μm^2 and compared (at 5 areas). The superficial layer and perforated surface of the meniscus were assessed by immunohistochemical analyses using anti-type I (Abcam, Cambridge, UK) and anti-type II (MP Biomedical, Solon, OH, USA) collagen antibodies as described.[29] Anti-type I and anti-type II collagen were used at 1:500 and 1:100 dilutions, respectively. Images were analyzed to quantify signal density using Image J software version 1.45 as previously described.[30] The mean value derived from 5 different images was evaluated by 2 observers. Intra- and interobserver reliabilities were excellent (ICC > 0.94).

Statistical analysis: Quantitative real-time PCR (four reactions for each cDNA sample), immunohistochemistry (3 chambers for each detection), migration assay (4 wells for each treatment) and proliferation assay (5 wells for each treatment) were repeated at least 3 times independently, and similar results were obtained. Data were expressed as means with standard deviations. Mean values were compared with one-way analysis of variance. Post-hoc comparisons were performed using the Holm–Sidak test. Significance was set at $P < 0.05$.

Results

HA treatment stimulates the proliferation of meniscus cells

Cell proliferation of both inner and outer cells was enhanced in a HA concentration-dependent manner. With HA concentration of 1000 μg/mL, inner cell proliferation was 1.16 times greater than that with 0 μg/mL HA. , and that with 100 μg/mL HA was 1.11 times than 0 μg/mL control (Fig. 1A). In contrast, outer cell proliferation with a HA concentration of 1000 μg/mL was 1.27 times than that with 0 μg/mL HA (Fig. 1B).

HA increases the migration of meniscus cells

The migration of inner meniscus cells increased with the addition of HA. Compared with HA concentration of 0 μg/mL, the migration of inner cells increased significantly in the 10, 100, and 1000 μg/mL groups (Fig. 2A), and the migration of outer meniscus cells significantly increased in the 100 and 1000 μg/mL groups (Fig. 2B).

HA enhances the expression of COL2A1 in inner meniscus cells

HA did not have any effect on COL1A1 gene expression in either the inner or outer cells (Fig. 3A). By contrast, although HA concentration-dependent increase in COL2A1 expression was

observed in the inner cells, no gene expression of COL2A1 was observed in the outer cells (Fig. 3A). Quantitative real-time PCR analyses revealed that COL2A1 gene expression significantly increased by HA treatments (100 and 1000 μg/mL) compared with HA-free condition ($P < 0.05$) (Fig. 3B). COL2A1 gene expression in the outer cells was not affected by HA supplementation (Fig. 3C).

HA increases the cell number on the perforated surface of organ-cultured meniscus

HA-treated groups showed no healing responses in macroscopic observations (Fig. 4A and B). On the perforated surface of the organ-cultured meniscal tissue, an increase in cell number was observed in the HA-treated group in both the inner and outer regions (Fig. 4C, D, G, and H). In the superficial layer, neither the inner nor outer region showed any change in cell number even with HA treatment (Fig. 4E, F, I, and J). Fibroblastic cells were observed in the perforated surfaces of both the inner and outer regions (Fig. 4C, D, G, and H). In both the inner and outer regions, HA treatments (1000 μg/mL) increased cellular counts on the perforated surfaces (Fig. 5A and B). The inner and outer regions of the superficial layer showed no change in cell counts (Fig. 5). In the adjacent area of the perforated surface of the inner region, HA treatment resulted in significantly higher reduction in the number of cells than those without HA treatment (Fig. 5C). Although no significant difference was found in the adjacent area of perforated surface of the

outer region, the number of cells was lesser in the HA treatment and without HA treatment (Fig. 5D). No significant difference was found in the adjacent area of the perforated surface of both the inner and outer regions (Fig. 5C and D).

HA induces the accumulation of type II but not type I collagen around the injured site of the meniscus

Type I collagen deposition was not influenced by HA treatment in either the perforated surface or the superficial layer of the meniscus (Fig. 6, A-H). By contrast, the perforated surface demonstrated strong staining pattern with anti-type II collagen antibody after HA treatment (Fig. 7, A-H). According to image analysis, the staining density with anti-type I collagen antibody showed no significant difference between with and without HA treatment (Fig. 8A and B), whereas the staining density with anti-type II collagen antibody in the perforated surface of both the inner and outer regions was significantly enhanced with HA treatment (Fig. 8C and D).

Discussion

In this study, the meniscus was separated into the inner and outer regions to examine the changes caused by HA treatment. The proliferative and migratory abilities of both the inner and outer cells were similarly enhanced by HA. HA also enhanced COL2A1 gene expression in the inner

meniscus cells. Nakata et al. examined meniscus cell proliferation at several concentrations of HA and observed concentration-dependent effects on cell proliferation.[31] Ito et al. reported that HA treatment enhanced the migratory ability of proximal tubule cells.[5] In our study, cell proliferation and migratory ability were activated in a HA concentration-dependent manner in both the inner and outer cells. HA promotes cell proliferation, increases volume and surface area for cell migration and cellular activities, and stimulates receptor-mediated events involving CD44, RHAMM, and ICAM-1.[32] These receptors forms a link with extracellular signal-regulated protein kinase (ERK), $P125^{fak}$, and $pp60^{c\text{-}src}$. The present study did not investigate these receptors. However, our results indicate the possibility that the healing process of meniscus injuries could be accelerated by HA treatment.

In the organ-culture model, no cell proliferation was induced in the superficial layers of either the inner region or the outer region. However, the increase of cell counts was observed on the perforated surface after HA treatment, and cell counts decreased in the adjacent area of the perforated surface in the inner region after HA treatment. Whether the cells on the perforated surfaces increased in number or the meniscus cells migrated remains unclear. Extracellular HA leads to its intracellular degradation via CD44 receptors.[33] HA accumulation coincides with cell migration and stimulates cell proliferation in vitro.[32] In our study, the superficial and perforated layers were both exposed to high HA concentration, but increased cell counts were observed only

on the perforated surface after HA treatment. We hypothesized that unless cells are exposed from the ECM due to events such as injury, the effects of HA may not be elicited.

No effects of HA on type I collagen syntheses were detected in both the superficial layer and perforated surface of organ-cultured meniscal samples. HA was directly linked to the cytokine-transforming growth factor-β1-dependent response.[34, 35] Transforming growth factor-β1 promotes the synthesis of type II collagen and aggrecan in human meniscus cells.[39] In addition, the majority of meniscal ECM consists of type I collagen. The ratio of type II collagen to type I collagen is 1:9[18], which may have contributed to the finding that HA had no effect on the increase of type I collagen production. Based on these findings, HA treatment did not lead to a detectable significant increase in its production, whereas HA enhanced type II collagen deposition. In a previous study, HA enhanced the expression of chondrogenic genes and blocked the expression of fibrogenic genes in hyaline cartilage.[36] Because the inner cells have a chondrocyte-like morphology[20], we presumed that the effect of HA on the meniscus is similar to that on chondrocytes.

This study has several limitations. HA with a mean molecular weight of 900,000 (630–1,170 kDa) was used in this study. The effects of NaHA have been reported to depend on the molecular size in vitro.[37] However, other references have indicated opposing findings in vivo.[37, 38] Because a low-molecular-weight HA was used in the present study, comparison with a

high-molecular-weight HA remains to be performed. Furthermore, changes in intracellular signals by means of HA treatment were not examined. Although we observed that cell proliferative and migration abilities of the inner and outer cells and COL2A1 gene expression of inner cells were enhanced by HA, we did not examine the underlying mechanism of these phenomena, which remains a question of future research. Finally, the study samples were older adults, showing osteoarthritis effects. However, we selected the patients with sufficient articular cartilage of the lateral femoral condyle and undegenerated lateral meniscus on magnetic resonance imaging. In addition, the lateral meniscus was macroscopically intact. Further investigations using healthy menisci or animal models will be required to understand the behavior of meniscus cells at the surface of injured menisci.

In conclusion, this study demonstrated that HA enhanced the proliferation and migration abilities of both inner and outer meniscus cells, whereas an increase in type II collagen expression could be observed in inner meniscus cells. HA supplementation may possibly promote the healing of the inner meniscus by inducing the proliferation, migration, and type II collagen synthesis of inner meniscus cells.

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Declaration of interest:

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Figure legends

Figure 1. HA treatment stimulated the proliferation of both the inner and outer meniscus cells. Compared with HA concentration of 0 µg/mL, a maximum 1.16-fold increase in the proliferation of inner meniscus cells was observed with the concentrations (10 to 1000 µg/mL) of HA treatment (A). Compared with HA concentration of 0 µg/mL, a maximum 1.27-fold increase in the proliferation of outer meniscus cells was observed with the concentration (1000 µg/mL) of HA treatment (B). *P < 0.05 compared with 0 µg/mL control.

Figure 2. HA increased the migration of the inner and outer meniscus cells. Compared with HA concentration of 0 µg/mL, a maximum 2.0-fold increase in the migration of inner meniscus cells was observed with the concentrations (10 to 1000 µg/mL) of HA treatment (A). Compared with HA concentration of 0 µg/mL, a maximum 3.1-fold increase in the migration of outer meniscus cells was observed with the concentration (1000 µg/mL) of HA treatment (B). *P < 0.05 compared with 0 µg/mL control.

Figure 3. HA treatment did not affect COL1A1 gene expression in cultured meniscus cells. HA enhanced COL2A1 expression in the inner meniscus cells but not outer meniscus cells (A).

Compared with HA concentration of 0 µg/mL, a maximum 2.4-fold increase in COL2A1 gene expression was observed with HA concentration of 1000 µg/mL (B). By contrast, no significant difference was observed in the outer meniscus cells (C). *P < 0.05 compared with 0 µg/mL control.

Figure 4. Meniscal fragments were fully penetrated with a 2-mm diameter punch at the 1/4th of the inner and outer regions (A and B). Meniscal samples were cultured for 3 weeks in a medium containing HA (0 and 1000 µg/mL). White arrowheads, perforated surfaces. Black arrowheads, superficial layers. Bars, 1 cm (A and B). HA increased the cell number on the perforated surface of the inner and outer meniscus cells (C, D, G, and H). By contrast, the cell number in the superficial layers did not change with the HA treatment (E, F, I, and J). Fibroblastic cells were observed in the perforated surfaces of both the inner and outer regions (C, D, G, and H). Open arrowheads indicate the meniscus cells. Bars, 50 µm (C-J).

Figure 5. HA treatment increased cell counts on the perforated surface in both the inner and outer region (A and B). No change in the number of meniscus cells on the superficial layer was observed with HA supplementation (A and B). In the adjacent area (200 × 200 μm^2) of perforated surface of inner region, HA treatment resulted in significantly higher reduction in the number of

cells than without HA treatment (C). No significant difference was found in the adjacent area of the perforated surface of the outer region (D). No significant difference was found in the adjacent area of the superficial layer (C and D). *P < 0.05 compared with 0 μg/mL control.

Figure 6. Type I collagen deposition in the perforated meniscal samples. HA treatment did not affect the distribution of type I collagen at perforated surfaces and superficial layers. Immunohistological staining of the inner (A-D) and outer (E-H) regions using anti-type I collagen antibody. HA-free (A, C, E, and G) and HA-supplemented (B, D, F, and H) conditions. Inserted small panels indicate negative controls in the absence of the primary antibody (A, B, E, and F). Open arrowheads indicate the meniscus cells. Bars, 50 μm.

Figure 7. Type II collagen deposition in the perforated meniscal samples. HA induced the accumulation of type II collagen around the injured site of the inner and outer meniscus regions. Inner (A-D) and outer (E-H) regions stained with anti-type II collagen antibody. Inserted small panels indicate negative controls in the absence of the primary antibody (A, B, E, and F). Open arrowheads indicate the meniscus cells. Bars, 50 μm.

Figure 8. Relative density of collagen deposition in perforated meniscal samples. HA treatments

showed no difference in staining density of anti-type I collagen antibody between the inner and outer regions (A and B). However, a significant increase in the staining density of anti-type II collagen antibody on the perforated surface was observed (C and D). *P < 0.05 compared with 0 µg/mL control.

Supplemental Figure. Meniscal fragments (B) were obtained from the lateral meniscus (A). Perforated holes (2-mm diameter) were created in both the inner and outer regions (C). Coronal sections of perforated holes in the inner (D) and outer region (E). Bars, 1 cm.

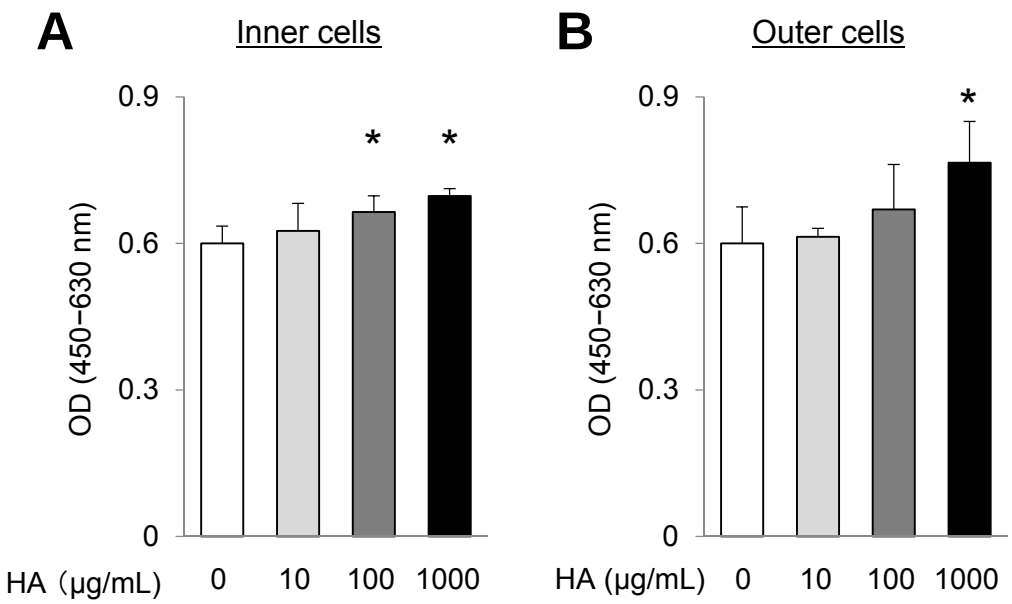


Fig. 1 Takaaki Tanaka

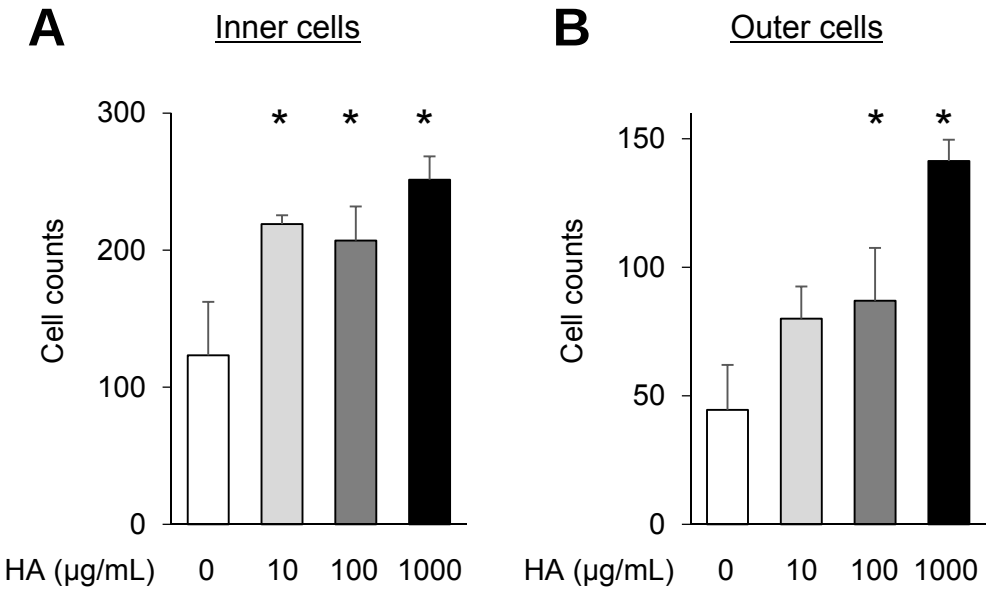


Fig. 2 Takaaki Tanaka

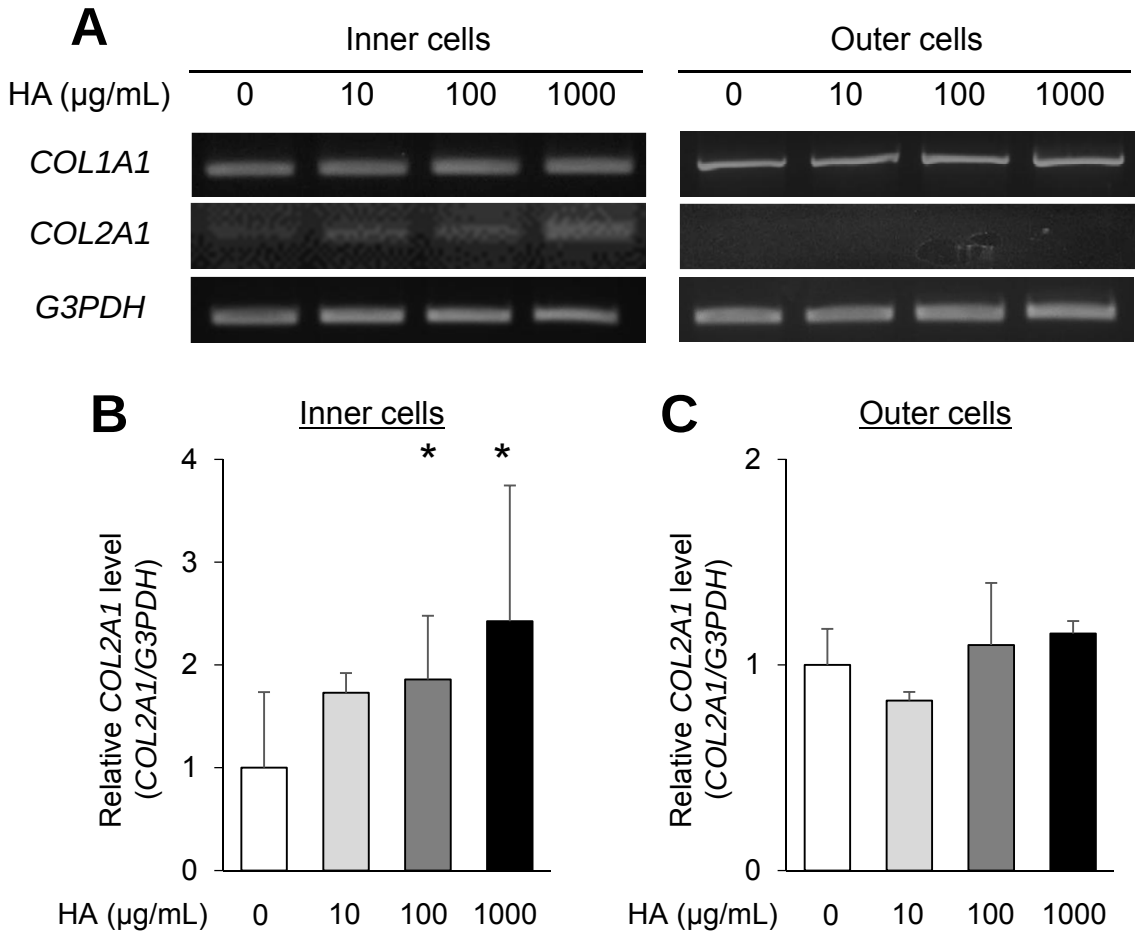


Fig. 3 Takaaki Tanaka

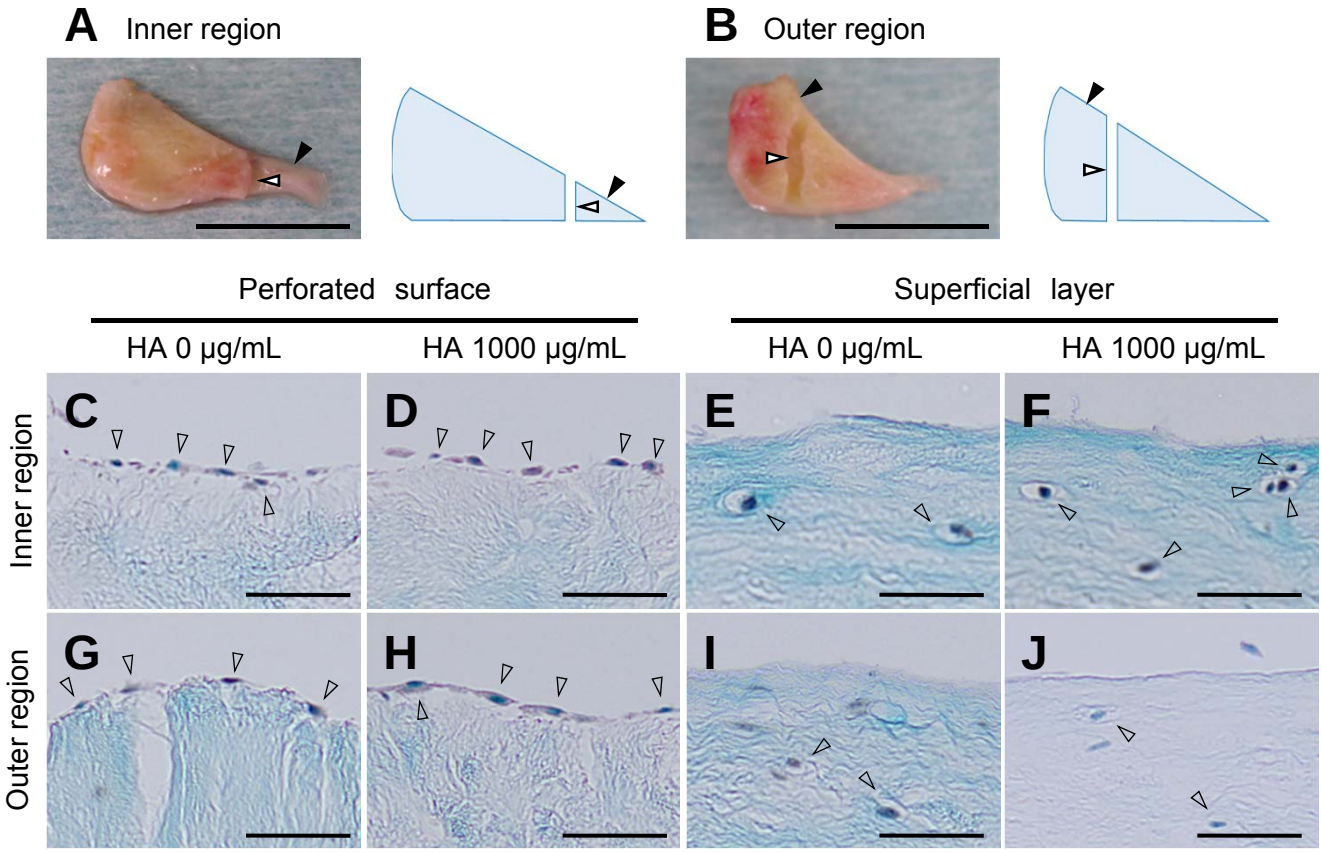


Fig. 4 Takaaki Tanaka

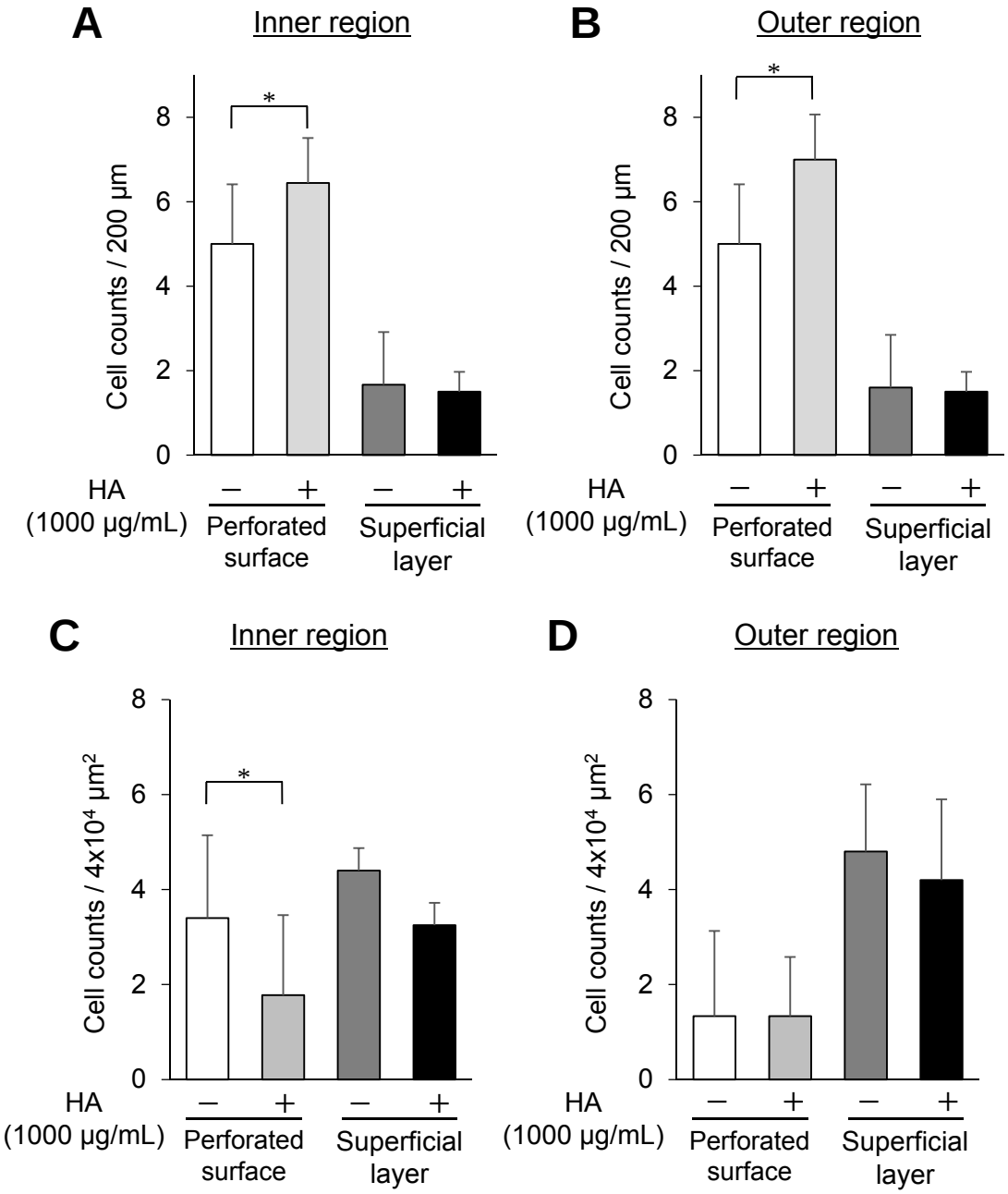


Fig. 5 Takaaki Tanaka

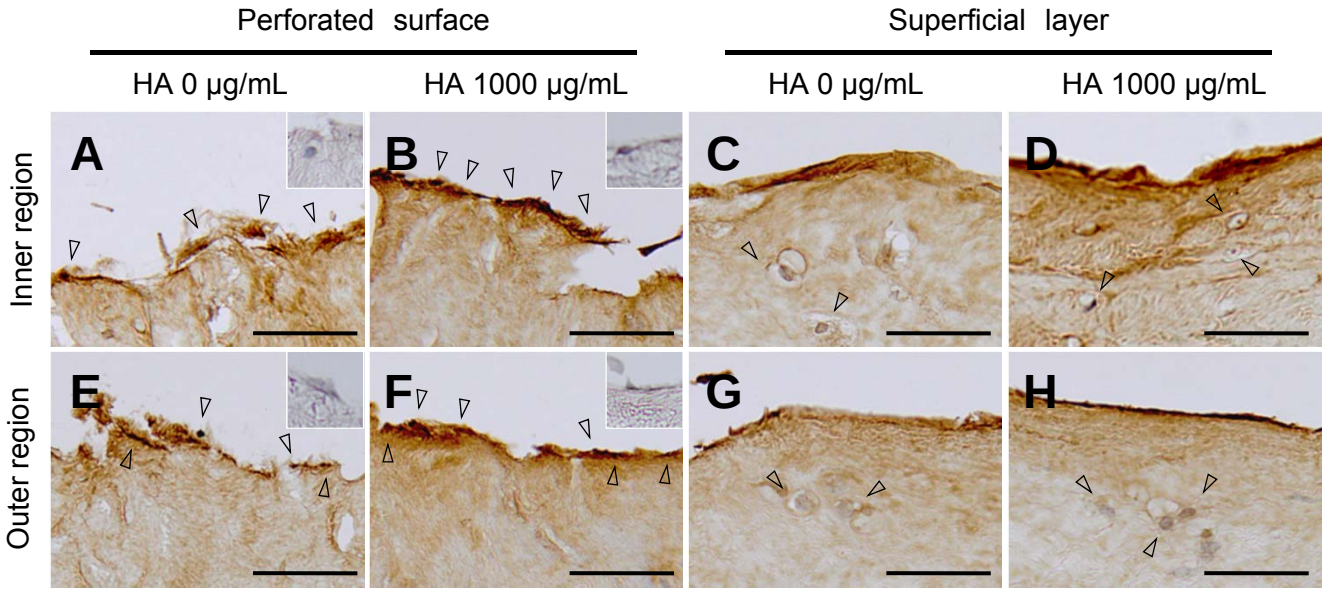


Fig. 6 Takaaki Tanaka

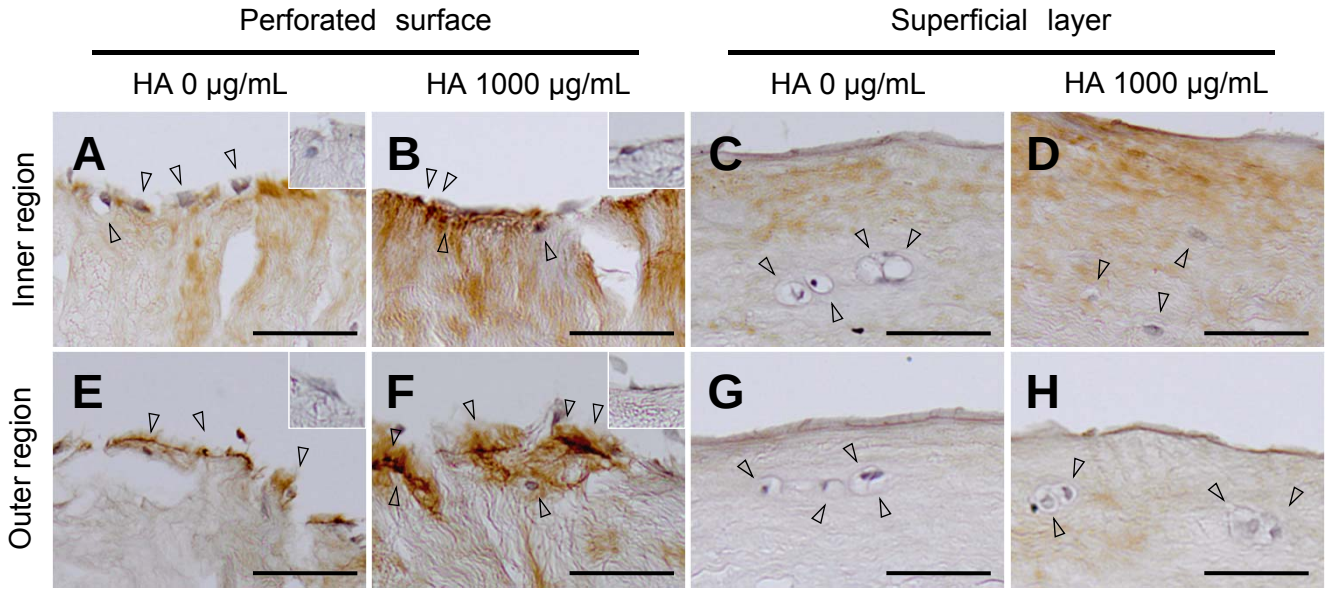


Fig. 7 Takaaki Tanaka

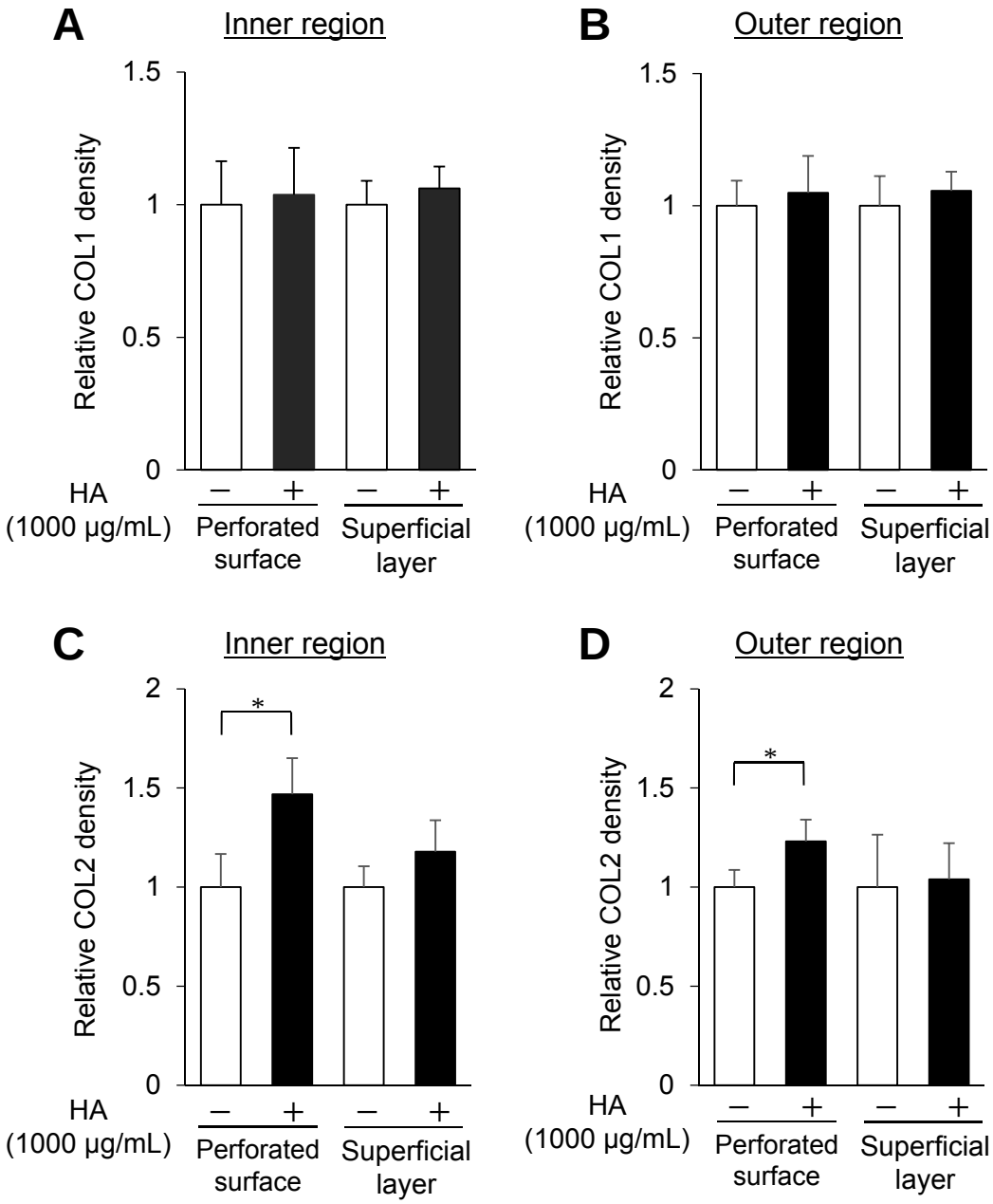
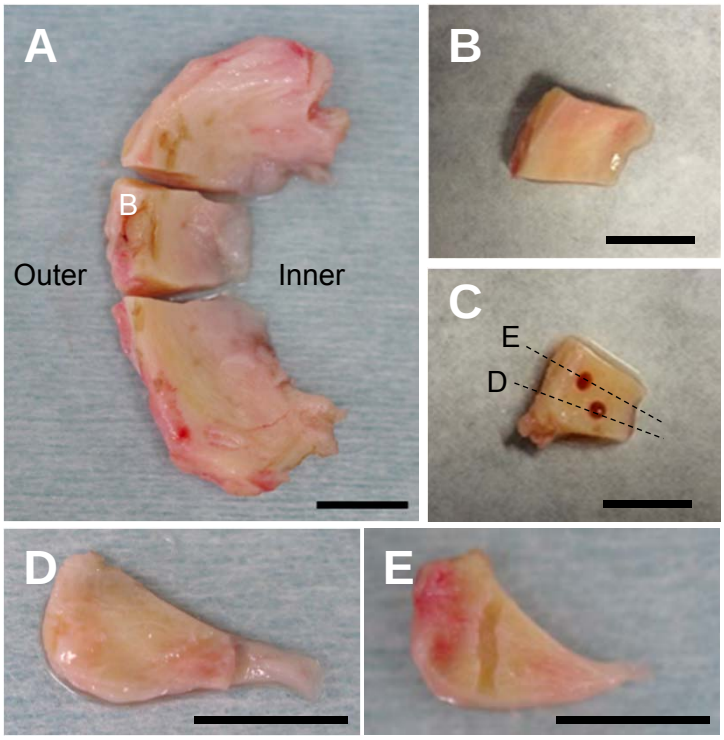


Fig. 8 Takaaki Tanaka



Supplemental Fig. Takaaki Tanaka