Self-assembled collagen fibrils from the swim bladder of Bester sturgeon enable alignment of MC3T3-E1 cells and enhance osteogenic differentiation

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Abstract

Collagen is the most abundant protein in animals, and its polymer, collagen fibrils, regulate cellular proliferation, differentiation, and migration. Low antigenicity, biocompatibility, and biodegradability make collagen fibrils suitable functional scaffolds for tissue engineering. In a previous study, we found that the type I atelocollagen purified from the swim bladder of Bester sturgeon (swim bladder collagen, SBC) showed high fibril-forming ability, producing thicker fibrils faster than porcine collagen. In this study, we report a novel method to coat cell culture wells with highly aligned collagen fibrils using the SBC. Two types of fibrils with different thicknesses were prepared by changing the crosslinking treatment timing. The oriented, thick collagen fibrils induced pre-osteoblastic MC3T3-E1, pre-adipocytic 3T3-L1, pre-myocytic C2C12, and fibroblastic L929 cells to align in the same direction, whereas the oriented, fine fibrils made a cell network with their long pseudopods. Cellular proliferation was inhibited on both fibrils. Furthermore, both fibrils induced the early differentiation of MC3T3-E1 cells without differentiation stimuli. In contrast, the morphology of pre-chondrocytic ATDC5 cells on both fine and thick fibrils extended very short pseudopods and continued to maintain a spherical shape without stretching, suggesting a distinct effect by the fibrils. The newly developed fibril coatings are in the form of a thin film, thereby providing good visibility of the cell structure, including cell-cell and cell-ECM interactions, using a phase contrast microscope. The fibril coatings have high potential as a useful tool for tissue engineering research.

Keywords: sturgeon collagen, swim bladder collagen (SBC), self-assemble, cell culture, cell orientation

1. Introduction

Collagen is the main component of the extracellular matrix (ECM) in animals and it mechanically supports tissues [1]. Type I collagen, which is found in skin, bones, ligaments, and other tissues, is secreted extracellularly as a triple helical molecule, assembles into fibrils with a diameter of 10–300 nm, and functions as a scaffold for cells [2,3]. Collagen is an excellent biomaterial for artificial scaffolds owing to its high cell adhesiveness, biocompatibility, and biodegradability, and is mainly used in the form of a hydrogel or sponge [4–6]. Recent reports suggest that soluble signaling molecules, such as hormones and cytokines, and insoluble ECMs, such as collagen, also control cell proliferation, differentiation, and migration by transmitting various information [7–10]. Therefore, scaffold materials that control cellular functions have been getting more attention as "smart scaffolds" in tissue engineering [11]. Notably, scaffolds made of atelocollagen are of researchers' interest because they can be introduced into the human body for medical application, even if they have a heterologous origin, because of its low antigenicity [12]. The scaffold needs to have appropriate morphology [13], stiffness [14], and amino acid sequences for cellular attachment [15,16] to adjust cellular functions. For example, the RGD sequence functions to induce cell orientation on collagen fibrils [10].

For mimicking the oriented fibrous form of ECM, such as observed in ligaments, synthetic polymers (e.g., polylactic acid, polyimide) with superior processing have been used with various technologies, such as electrospinning [17,18] and surface micropatterning [19,20]. Recent reports demonstrated the effects of fibril thickness on cellular behavior; for example, 70–100 nm diameter fibrils

promote specific differentiation of human MSCs into osteoblasts [21]. Few reports have described the effects of fibers with diameters on the order of nanometers, as is collagen *in vivo*. Additionally, it is difficult to mimic aligned collagen fibrils *in vivo* owing to the low processing ability of collagen fibrils *in vitro*. Several strategies have been developed to build highly-aligned fibrillar collagen structures, including electrospinning [22], flow casting [23], and high magnetic field [24,25]. For example, control of the fibril alignment by a magnetic field can produce thin diameter fibrils close to natural collagen fibrils. However, the high cost of the process may hinder industrial production and the risk of denaturation of collagen molecules during processing must be considered [26].

In recent years, the most common sources of collagen for biomaterials and tissue engineering have been bovine skin and tendon, porcine skin, and rat tail [27]. However, the use of mammalian collagens has the risk of zoonoses such as bovine spongiform encephalopathy and foot-and-mouth disease. Religious beliefs also restrict the usage of porcine or bovine collagen. Marine collagen derived from aquatic animals has attracted attention as a smart alternative to mammalian collagen owing to their low risk of pathogen infections and low religious restrictions. We observed that the swim bladder of the Bester sturgeon, a hybrid of *Huso huso* × *Acipenser ruthenus*, contains a large amount of type I collagen [28]. Because the Bester sturgeon is a highly valued fish species especially famous for its caviar and is now widely aquacultured in China and Japan, the industrial-scale production of the SBC is possible. In addition, the SBC is characterized by low viscosity, high fibril-forming ability, and relatively high denaturation temperature [28]. However, atelocollagens from other tissues (scale, skin, muscle, digestive tract,

notochord, and snout cartilage) of the Bester sturgeon do not show such properties [28]. The special characteristics of the SBC enable the production of materials with a uniform orientation of fibrils using very easy and low-cost techniques [29,30]. We assumed that the especially high fibril-forming ability of SBC made it possible to align the fibrils with uniform orientation.

In this study, we developed an easy procedure to coat the bottom of cell culture wells with highly oriented fibrils using the SBC, and examined how oriented fibrils affected cell morphology, proliferation, and differentiation.

2. Material and Methods

2.1. Materials

Type I atelocollagen was extracted from the swim bladder of Bester sturgeon as described previously [28]. Porcine pepsin (EC 3.4.23.1, 1:10,000, Wako Pure Chemical Industries Ltd., Osaka, Japan) at a concentration of 0.1% was used for removing telopeptides of collagen molecules. For comparison, porcine skin type I collagen (Cellmatrix type I-C, Nitta Gelatin Inc., Osaka, Japan) was used. Cell culture media and penicillin/streptomycin (P/S) solution were obtained from Thermo Fisher Scientific (Waltham, MA). Hank's balanced salt was obtained from Sigma-Aldrich (St. Louis, MO). Other chemicals were obtained from Wako Pure Chemical Industries Ltd unless otherwise stated. Cell culture wares were from Corning, NY. Cells were obtained from RIKEN Cell Bank (Tsukuba, Japan) and Japanese Collection of Research Bioresources (Tokyo, Japan).

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97 *2.2. Preparation of collagen-coated wells*

98 Lyophilized SBC was dissolved in an aqueous HCl solution (pH 3.0) at 1.0% (w/v), 4°C without stirring to
99 produce a homogenous solution. A 200 μl aliquot of the atelocollagen solution was poured into each well of
100 a 24-well cell culture plate, incubated for 30 minutes, and the solution was removed. The fibril formation
101 was induced by introducing a 0.1 M Na-phosphate buffer (PB, pH 7.6) into the wells for 24 hours at room
102 temperature (22°C). Crosslinking was performed using a 1 mM genipin in a 0.1 M PB for 24 hours to
103 prevent heat denaturation. Two ways of crosslinking were employed: crosslinking during fibril formation
104 and after fibril formation. In the former case, the PB containing genipin was used to induce fibril formation.
105 In the latter, wells were treated first with PB to induce fibril formation for 24 hours and then with PB
106 containing genipin for a subsequent 24 hours. After the crosslinking, wells were washed twice with cold
107 Hank's balanced salt solution (HBSS) containing 100 U/ml penicillin and 100 μg/ml streptomycin. For
108 comparison, wells were coated with porcine skin type I collagen following the instructions supplied by the
109 company. Briefly, collagen (0.03%, w/v) in an aqueous HCl solution was pipetted into the wells, incubated
110 for 30 minutes at room temperature (22°C), air-dried, crosslinked with PB containing 1 mM genipin for 24
111 hours, and rinsed twice with HBSS. SBC at a concentration of 0.03% was similarly coated. The genipin
112 crosslinking increases the thermal stability of the gels by the same degree as glutaraldehyde and is very low
113 toxicity [31]. 1 mM genipin was used for the crosslinking, under which no cell toxicity was observed [31].

114

115 *2.3. Cell culture*

116 MC3T3-E1, ATDC5, 3T3-L1, C2C12, and L929 cell lines, which showed pre-osteoblastic, pre-
117 chondrocytic, pre-adipocytic, pre-myocytic, and fibroblastic features, respectively, were used to evaluate
118 cellular response. To study the cellular morphology and proliferation, cells were maintained in their
119 respective growth media: Minimum Essential Medium Eagle (MEM)-alpha supplemented with 10% fetal
120 bovine serum (FBS) for the MC3T3-E1 cells, Dulbecco's Modified Eagles Medium/Nutrient Mixture F12
121 (DMEM/F12) supplemented with 10% FBS for the ATDC5 cells, DMEM supplemented with 10% FBS for
122 the 3T3-L1 and C2C12 cells, and MEM supplemented with 10% FBS for the L929 cells. To study
123 differentiation, MC3T3-E1 cells were seeded and maintained in the growth medium for one day and after
124 that cultured in the osteogenic medium, growth medium supplemented with 10 mM β-glycerophosphate
125 and 50 μM ascorbic acid (Sigma-Aldrich). All cells were cultured in a humidified incubator at 37°C and
126 5% CO_2, and media was changed once every three days. All cultures were supplemented with 100 U/ml
127 penicillin and 100 μg/ml streptomycin.

128 The cell morphology was observed with a phase contrast microscope (DMI600B, Leica, Wetzlar,
129 Germany) over the cell culture time.

130

131 *2.4 Scanning electron microscopy*

132 The microstructure of the bottom surface of the cell culture wells coated with the different collagens was
133 studied under a scanning electron microscope (SEM; JSM6010LA, JEOL Ltd., Tokyo, Japan). PB

containing 2.5% (v/v) glutaraldehyde was poured into each well and incubated for 3 hours at room temperature for fixation. The samples were then rinsed twice with PB, dehydrated with ethanol, and the medium was changed to a t-butyl alcohol solution. The samples in t-butyl alcohol were then freeze-dried with a freeze-drying device (JFD-320; JEOL Ltd.), and the sidewalls of each well were cut off. The cell culture bottom was coated with gold-platinum using an auto fine coater (JFC-1600; JEOL Ltd.) and observed and digitally imaged by the SEM. 100 fibrils were randomly selected and their diameters were determined with ImageJ software.

2.5. Cell proliferation analysis

The relative cell number was determined by the measurement of DNA content in the well. At the cell cultures endpoints, the wells were emptied of media and rinsed once with PB containing 0.8% NaCl (PBS). The wells were stored at -30°C. To perform the assay, the well plates were thawed and digested in a cell lysate buffer (0.1 mg/ml Proteinase K with 0.01% SDS in PBS). The DNA content of the sample was measured after appropriate dilution with PBS using a fluorescent plate reader (ARVO X, PerkinElmer, Waltham, MA) (excitation, 355 nm; emission, 460 nm) with the addition of Hoechst 33258 (2 μg/ml). DNA from salmon sperm was used as a standard.

2.6 Differentiation of MC3T3-E1 cells

The osteoblastic differentiation of cultured MC3T3-E1 cells was assessed by quantifying alkaline

phosphatase (ALP) activity, which is synthesized primarily at an early stage of bone differentiation by osteoblasts [32]. The cells were washed with PBS and digested in 0.05% Triton X-100 (MP Biomedicals Inc., Santa Ana, CA). After centrifugation at 21,000*g* for 15 min (Hitachi Koki, Tokyo, Japan), the supernatants were collected, and the ALP activity was quantified using a LabAssay ALP kit (Wako) following the manufacturer's instructions. The color density was determined at 405 nm by a plate reader (infiniteF50R, Wako).

2.7. Statistics

Data was analyzed by ANOVA and a Tukey–Kramer post hoc test using Microsoft Excel add-in statistical software for multiple comparisons (SSRI, Tokyo, Japan). Values were expressed as the mean ± standard error. Statistical significance was accepted at $P < 0.05$.

3. Results

3.1. SEM images of collagen-coated wells

When the porcine collagen or SBC solution at a concentration of 0.03% (w/v) was coated on the bottom of the well, several aggregates of collagen molecules were observed, but no fibrils were confirmed (Figs. 1A, B). Thus, we concluded that the bottom was coated with collagen molecules. Hereafter, the coating method is called "molecular coating" and the coated wells "molecule-coated wells". When the well was coated with a 1% SBC solution, aligned collagen fibrils appeared. By changing the timing of the crosslinking treatment

with genipin, two types of fibril-coated wells showing different fibril thickness were obtained (Figs. 1C, D). When fibrillogenesis and crosslinking were conducted simultaneously for 24 hours using the PB containing genipin, thin fibrils were aligned in the same direction on the well (Fig. 1C). In contrast, when fibrillogenesis for 24 hours and crosslinking for 24 hours were conducted sequentially, thick fibrils overlaid the thin fibrils (Fig.1D). Both the thick and thin fibrils were aligned in the same direction, but the direction uniformity was greater in the thin fibrils. The diameter range of the simultaneously crosslinked fibrils was 51–192 nm with a mean diameter of 99.0 ± 2.8 nm, while the diameter range of the sequentially crosslinked fibrils was 53–520 nm with a mean diameter of 178.2 ± 8.1 nm (Fig. 2). Hereafter, we call the simultaneously and sequentially crosslinked coating methods as “fine-fibril coating” and “coarse-fibril coating”, respectively, and the coated wells as “fine-fibril-coated wells” and “coarse-fibril-coated wells”, respectively.

3.2. Cell morphology

The cell morphology was examined in the growth media appropriate to each cell type. All cells used in the present study showed good adhesion to all substrates. When the cells were well adhered, their morphologies were generally similar, except for the ATDC5 cells. This paper provides the morphologies of the MC3T3-E1 (Fig. 3) and ATDC5 (Fig. 4) cells as representative examples. The morphologies of 3T3-L1, C2C12, and L929 cells are supplied as Supplementary Materials (Suppl. Figs. 1-3).

The SBC molecule-coated wells render MC3T3-E1 cells faster adhesion and extension than the

191 porcine collagen-molecule-coated wells (Figs. 3A, D). Fast cell adhesion on the SBC-molecule-coated well
192 was observed similarly with or without crosslinking with genipin (data not shown). A similar tendency was
193 also observed in other cell types. The cells on the fibril collagen immediately adhered to the surfaces like
194 on the SBC molecules (Figs. 3G, J). Before confluency, the MC3T3-E1 cells on the porcine skin collagen
195 and SBC molecule-coated wells showed spindle or polygonal shapes, representing typical fibroblastic
196 morphology (Figs. 3B, E). On the fine-fibril-coated well, the cell bodies were elongated but small
197 compared with those on the molecule-coated wells (Figs. 3H). The cells extended their pseudopod to form
198 a network (Fig. 3H). On the coarse-fibril-coated well, the shape of the cell bodies was similar to that of the
199 fine-fibril-coated well, but the cell extensions were aligned in the same direction (Fig. 3K). When cells
200 reached confluency, the cell shape became a spindle on the molecule-coated wells (Figs. 3C, F). Cells on
201 the fine- and coarse-fibril-coated wells increased their numbers but did not reach confluency at seven days
202 of culture (Figs. 3I, L).

203 ATDC5 pre-chondrocytes seeded on the porcine skin collagen and SBC molecule-coated wells
204 showed spindle or polygonal shapes and reached confluency after seven days (Figs. 4-F). The morphology
205 of ATDC5 cells on the fibril-coated wells was clearly different than the other cell types; they extended very
206 short pseudopods on both fine- and coarse-fibril-coated wells one day after seeding (Figs. 4G, J) and
207 continued to maintain a spherical shape without stretching until the end of the culture (seven days),
208 although the cell density increased (Figs. 4H, I, K, L).

209 In this study, collagen fibrils of SBC were crosslinked with genipin for application to cell

cultures. Without genipin crosslinking, denaturation of collagen fibrils gradually occurred during cell culture, and the MC3T3-E1 cells on the coarse-fibril-coated well lost their orientation in association with the loss of collagen fibril orientation (Suppl. Fig. 4).

3.3. Cell proliferation

Cell proliferation was examined using growth media appropriate to each cell type. The MC3T3-E1 cells on both fine- and coarse-fibril-coated wells proliferated slowly compared with molecule-coated wells (Fig. 5A). The proliferation on the coarse-fibril-coated well was the slowest. Because no adverse effects of genipin crosslinking on the proliferation of cells on the molecule-coated wells were observed (data not shown), the slower cellular proliferation on the fine- and coarse-fibril-coated wells is due to the collagen fibrils. Similarly, in the ATDC5 cells, proliferation was slow on the fibril-coated wells (Fig. 5B). Fibrils commonly inhibited proliferation of all cell types used in this study (Suppl. Fig. 5).

3.4. Cell differentiation

The ALP activity was measured as an early marker for osteoblast differentiation. In this study, genipin treatment was not conducted on the molecule-coated wells. Cells were cultured in a growth medium for one day after seeding and then differentiation was stimulated. The assay was conducted just before stimulation of differentiation (Day 1), and two and six days after the stimulation (Days 3 and 7).

Cells on both porcine collagen and SBC molecule-coated wells had increased ALP activities on

Day 7 (Fig. 6). There were no significant differences between these two groups. In contrast, the ALP activities of cells on the fine- and coarse-fibril-coated wells showed the greatest values on Day 1 and decreased thereafter (Fig. 6). At all days, the ALP values of the cells on the fine-fibril-coated groups were the highest, followed by those of the coarse-fibril-coated groups, and the values of the porcine collagen and SBC molecule-coated groups were the lowest.

4. Discussion

In this study, we developed a novel method to coat aligned collagen fibrils on the bottom of cell culture wells. Techniques such as micropatterning are conventionally used to control the behavior of cells and to arrange the orientation of cell stretching. However, methods applicable to biomaterials such as collagen are very limited, and it is extremely difficult to industrially produce large quantities of materials made of aligned collagen fibrils. SBC produced highly-oriented collagen fibrils on the bottom of cell culture wells using a very simple process. By changing the timing of crosslinking treatment with genipin, two types of fibril-coated wells with different fibril thickness were obtained. The ECM topography [33], elasticity [34], and physical/gravitational stimulation caused by movement [35] are recently recognized as important cellular regulators. The ECM also regulates cell morphology: cells can recognize topography of the ECM and align along the fibers of an oriented fiber structure [19,20,36,37]. Therefore, fibrils oriented on a well must have regulatory cues for cell morphology and functions to reproduce an ECM environment closer to that of tissues in the animal body such as lamellar bones, tendons, and ligaments. Additionally, the newly

developed fibril-coatings are in the form of a thin film, thereby providing good visibility of the cell structure, including cell-cell and cell-ECM interactions, as observed by phase-contrast microscopy.

The present study showed that the SBC fibrils on the bottom of cell culture wells aligned in the same direction in the fibril coatings. We found that the orientation was similar to the flow direction of the PB that was introduced to the wells to force fibrillogenesis. The shear force occurred at the interface of the SBC and the PB when the PB is poured into the well is the possible mechanism for the alignment of fibrils into the same direction. This phenomenon may occur because the SBC rapidly make fibrils under the neutral pH since the introduction of the PB to the culture well took only several seconds. The similar alignment of the fibrils occurred when the 4% SBC solution was injected from a pipet to the buffer solution [30]. In addition, the low viscosity of the SBC solution is needed to conduct the coatings using a concentration of 1% SBC solution. Thus, the special properties of the SBC, such as fast fibril formation, high solubility, and low viscosity, make it possible to coat highly-oriented collagen fibrils. Additionally, our preliminary experiments using a transmission electron microscope (TEM) showed that the SBC fibrils formed in a test tube had the D-periodic pattern (data not shown); thus, the SBC fibrils formed *in vitro* retain the structure close to that of collagen fibrils *in vivo*. Although the TEM observation of the SBC fibrils coated on the bottom surface of the well has not conducted yet, it is possible that the coated SBC fibrils also retain the D-periodic structure.

In the preliminary experiments, we revealed that the bottom of the cell culture well was partially coated with fibrils when the concentration of the collagen solution is 0.5%, whereas, at 3%, the bottom was

thickly coated with fibrils (data not shown). Therefore, the concentration of the collagen solution is the regulatory key for the structure of the collagen on the bottom. At the concentration of 0.03% for the molecular coating, the collagen concentration is too low, and thus, the density of collagen molecules may be too low to form fibrils.

This study revealed that cells on the fine- and coarse-fibril-coated wells showed distinct morphologies: the cells on the fine fibrils made a network extending their pseudopods, whereas the cells on the coarse fibrils became aligned. Thus, one of the cues that fibrils give to the cells actually relates to cell morphology. We speculate that differences in the fibril thickness cause these morphological differences. Since the fibril diameter (ca. 99 nm) of the fine-fibril coating is close to the minimum fiber thickness for cell orientation [38], cells on the fine fibrils may not become aligned completely due to slight diameter variation. In other words, the thick fibrils of the coarse-fibril coating may be important for ensuring the orientation of the cells. Using the SEM, Zhang et al. [28] observed that a fusion of several thin fibrils made a thick fibril. In their experiment, no genipin crosslinking was conducted. Thus, under the condition making the fine-fibril coating, the genipin crosslinking that occurred simultaneously with the fibrillogenesis may inhibit the fusion of thin fibrils.

Cell morphology is an important factor for understanding cell properties and functions. The size of cell bodies on the fine- and coarse-fibril coatings tend to be smaller than those on molecular coatings, and thus, the cells changed their steric structure closer to that *in vivo*. In addition, the cells on the fine-fibril coating had extremely long pseudopods connecting each other, while the cells on the coarse-fibril coating

286 became aligned. The integrin-mediated cell adhesion to the scaffold with different topographical characters
287 may cause such morphological difference. Integrin is a transmembrane receptor that mediates cell
288 attachment to the ECM and activates focal adhesion kinase (FAK). Activation of the FAK affects cell
289 migration and cell proliferation in the presence of growth factor-related signals [39]. Integrin also regulates
290 the responsiveness of growth factor receptors and the apoptotic pathway of cells as a scaffold for adhesion-
291 dependent signal transduction [40]. Although the growth factors provide the trigger for cell proliferation, it
292 is the integrin-mediated cell adhesion that sustains the signal of the growth factors [41]. As described
293 below, distinct cell proliferation and differentiation patterns were observed when cells were cultured on the
294 fine- and coarse-fibril-coatings. Such responses would be induced by a consequence of the collaboration of
295 signals from growth factors and integrins.

296 The present study revealed the general suppression of cell proliferation of MC3T3-E1 and
297 ATDC5 cells on fine- and coarse-fibril-coated wells. Alternatively, the ALP activity of MC3T3-E1 cells
298 cultured on the fibril-coated wells showed very high values one day after culture in the growth medium.
299 This result indicates that osteogenic differentiation was promoted by SBC fibrils without any stimuli for
300 differentiation in the culture medium. The results corroborate past reports that osteogenic differentiation is
301 promoted by culturing cells on the fibrous structures [19,20,42,43].

302 The collagen fibril diameter of bone is 50–300 nm *in vivo* [44], and Watari et al. [20] reported
303 that oriented 200 nm diameter fibers most effectively induced osteogenic differentiation. In the present
304 study, the fine-fibril-coating (fibril diameter 51–192 nm) more effectively induced ALP activity than the

coarse-fibril-coating (fibril diameter 53–520 nm). Therefore, thicker fibrils of the coarse-fibril coating may inhibit osteoblastic differentiation compared with fine fibrils. It is also possible that the fibril diameter and other factors that are presently unknown might affect osteogenic differentiation on fibril coatings.

The activation of the ALP activity on the fibril coatings was, however, a transient phenomenon: it gradually decreased with prolonged culture in the osteogenic medium. This result implicates that the differentiation stage of the MC3T3-E1 cells proceeded with time because ALP is an early differentiation marker of osteoblasts. However, the precise mechanisms why ALP activity was transiently activated remain to be clarified. Similar transient activation of gene expressions short term after seeding was also reported in human fibroblasts on a nanoscale groove structure (width, 12.5 μm; depth, 2 μm): the expression of genes involved in the cytoskeleton as well as signaling, transcription, and translation greatly increased at Day 1 and then decreased at Day 5 [33]. Although the authors suggested the possibility of the topographical structure for affecting the cellular transcription and translation, they do not describe the reason for the transient effects [33].

Unlike other cell types used in this study, ATDC5, a chondrogenic cell line, maintained its spherical shape on the fine- and coarse-fibril-coated wells for at least seven days during the culture. This morphology is seen in differentiated chondrocytes in animal, raising the possibility of promoted differentiation. Abagnale et al. [19] reported that both osteogenic and adipocytic differentiation, which are mutually exclusive, were promoted with submicroscale fibrous structures. Consequently, the fibril coatings may promote osteogenic differentiation and the differentiation of other cell types. It is necessary to clarify

whether the SBC fibrils promote the differentiation of stem cells into single or plural lineages.

Cells bind to collagen using integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$, and $\alpha_{11}\beta_1$ [46]. The specific amino acid sequence, glycine-phenylalanine-hydroxyproline-glycine-glutamic acid-arginine (GFOGER), of collagen triple-helical molecule was reported as the binding site of integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_{11}\beta_1$ [15, 46]. Thus, cells on the culture wells in this study use these integrins and bind to the GFOGER motifs of the SBC molecules, porcine collagen-molecules, and SBC fibrils. It is also possible that cells additionally use the general integrin-binding motif, an arginine-glycine-aspartic acid (RGD) sequence, of collagen. The present study revealed that the adhesion and extension of MC3T3-E1 cells on the SBC molecule-coated wells were faster than those on the porcine collagen-molecule-coated well. Also, the adhesion of the cells to SBC fibrils was fast. The precise mechanism of the better cellular adhesion and extension of the SBC is yet unknown, but we hypothesize that a greater number or accessibility of GFOGER and RGD motifs in SBC may determine better cellular adhesion and extension. However, mRNA sequences and deduced amino acid sequences of α-chains in sturgeon were only incompletely conducted; Zhang [47] and Zhang et al. [48] reported complete cDNA sequences of α1- and α2-chains of type I collagen from Amur sturgeon, but the sequence of a fish-specific α-chain (α3) of the sturgeon were not determined. To prove the hypothesis, the priority of the future study is on the cDNA cloning of the α3-chain.

5. Conclusion

In tissue engineering research and application, biological scaffolds that control cell proliferation and differentiation have attracted attention [11]. Since ECM with collagen transmits various information to cells and regulates cell proliferation and differentiation [7,9,45], we established a unique method that produces two types of highly-aligned collagen fibrils on cell culture wells using SBC without any special technology, revealing that osteoblasts on the fibril coatings showed a low growth rate with accelerated osteogenic differentiation. The newly developed fibril coatings are in the form of a thin film, thereby providing good visibility of the cell structure, including cell-cell and cell-ECM interactions, under a phase-contrast microscope. Thus, the fibril coatings have high potential as a tool for tissue engineering research. Additionally, the coating will be applicable surface modifications of artificial scaffolds made of synthetic polymers to increase their cellular adhesiveness and biocompatibility.

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357 References

358 [1] J.C. Adams, F.M. Watt, Regulation of development and differentiation by the extracellular matrix,

359 Development. 117 (1993) 1183–1198.

360 [2] K.E. Kadler, D.F. Holmes, J.A. Trotter, J.A. Chapman, Collagen fibril formation, Biochem. J. 316

361 (Pt 1) (1996) 1–11.

362 [3] D.J. Prockop, Hulmes David J. S., Assembly of Collagen Fibrils, Elsevier Science, 1994.

363 [4] S.-A. Oh, H.-Y. Lee, J.H. Lee, T.-H. Kim, J.-H. Jang, H.-W. Kim, I. Wall, Collagen Three-

364 Dimensional Hydrogel Matrix Carrying Basic Fibroblast Growth Factor for the Cultivation of

365 Mesenchymal Stem Cells and Osteogenic Differentiation, Tissue Eng. Part A. 18 (2012) 1087–

366 1100.

367 [5] J. Glowacki, S. Mizuno, Collagen scaffolds for tissue engineering, Biopolymers. 89 (2008) 338–

368 344.

369 [6] B. V. Slaughter, S.S. Khurshid, O.Z. Fisher, A. Khademhosseini, N.A. Peppas, Hydrogels in

370 Regenerative Medicine, Adv. Mater. 21 (2009) 3307–3329.

371 [7] D. Gospodarowicz, D. Delgado, I. Vlodavsky, Permissive effect of the extracellular matrix on cell

372 proliferation in vitro, Proc. Natl. Acad. Sci. U. S. A. 77 (1980) 4094–8.

373 [8] H. Koyama, E.W. Raines, K.E. Bornfeldt, J.M. Roberts, R. Ross, Fibrillar Collagen Inhibits Arterial

374 Smooth Muscle Proliferation through Regulation of Cdk2 Inhibitors, Cell. 87 (1996) 1069–1078.

375 [9] M. Mizuno, R. Fujisawa, Y. Kuboki, Type I collagen-induced osteoblastic differentiation of bone-

marrow cells mediated by collagen-α2β1 integrin interaction, J. Cell. Physiol. 184 (2000) 207–213.

[10] K. Poole, K. Khairy, J. Friedrichs, C. Franz, D.A. Cisneros, J. Howard, D. Mueller, Molecular-scale Topographic Cues Induce the Orientation and Directional Movement of Fibroblasts on Two-dimensional Collagen Surfaces, J. Mol. Biol. 349 (2005) 380–386.

[11] D.G. Anderson, J.A. Burdick, R. Langer, Smart biomaterials, Science. 305 (2004) 1923–4.

[12] B. Pontz, W. Meigel, J. Rauterberg, K. Kuhn, Localization of Two Species Specific Antigenic Determinants on the Peptide Chains of Calf Skin Collagen, Eur. J. Biochem. 16 (1970) 50–54.

[13] G.T. Christopherson, H. Song, H.-Q. Mao, The influence of fiber diameter of electrospun substrates on neural stem cell differentiation and proliferation, Biomaterials. 30 (2009) 556–564.

[14] M. Levy-Mishali, J. Zoldan, S. Levenberg, Effect of Scaffold Stiffness on Myoblast Differentiation, Tissue Eng. Part A. 15 (2009) 935–944.

[15] C.G. Knight, L.F. Morton, A.R. Peachey, D.S. Tuckwell, R.W. Farndale, M.J. Barnes, The collagen-binding A-domains of integrins α1β1 and α2β1 recognize the same specific amino acid sequence, GFOGER, in native (triple-helical) collagens, J. Biol. Chem. 275 (2000) 35–40.

[16] H. Shin, S. Jo, A.G. Mikos, Biomimetic materials for tissue engineering, Biomaterials. 24 (2003) 4353–4364.

[17] R.D. Cardwell, L.A. Dahlgren, A.S. Goldstein, Electrospun fibre diameter, not alignment, affects mesenchymal stem cell differentiation into the tendon/ligament lineage, J. Tissue Eng. Regen. Med. 8 (2014) 937–945.

[18] F. Yang, R. Murugan, S. Wang, S. Ramakrishna, Electrospinning of nano/micro scale poly(l-lactic acid) aligned fibers and their potential in neural tissue engineering, Biomaterials. 26 (2005) 2603–2610.

[19] G. Abagnale, M. Steger, V.H. Nguyen, N. Hersch, A. Sechi, S. Joussen, B. Denecke, R. Merkel, B. Hoffmann, A. Dreser, U. Schnakenberg, A. Gillner, W. Wagner, Surface topography enhances differentiation of mesenchymal stem cells towards osteogenic and adipogenic lineages, Biomaterials. 61 (2015) 316–326.

[20] S. Watari, K. Hayashi, J.A. Wood, P. Russell, P.F. Nealey, C.J. Murphy, D.C. Genetos, Modulation of osteogenic differentiation in hMSCs cells by submicron topographically-patterned ridges and grooves, Biomaterials. 33 (2012) 128–136.

[21] S. Oh, K.S. Brammer, Y.S.J. Li, D. Teng, A.J. Engler, S. Chien, S. Jin, Stem cell fate dictated solely by altered nanotube dimension, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 2130–5.

[22] J.A. Matthews, G.E. Wnek, D.G. Simpson, G.L. Bowlin, Electrospinning of collagen nanofibers, Biomacromolecules. 3 (2002) 232–238.

[23] Y. Tanaka, K. Baba, T.J. Duncan, A. Kubota, T. Asahi, A.J. Quantock, M. Yamato, T. Okano, K. Nishida, Transparent, tough collagen laminates prepared by oriented flow casting, multi-cyclic vitrification and chemical cross-linking, Biomaterials. 32 (2011) 3358–3366.

[24] N. Builles, H. Janin-Manificat, M. Malbouyres, V. Justin, M.-R. Rovère, G. Pellegrini, J. Torbet, D.J.S. Hulmes, C. Burillon, O. Damour, F. Ruggiero, Use of magnetically oriented orthogonal

collagen scaffolds for hemi-corneal reconstruction and regeneration, Biomaterials. 31 (2010) 8313–8322.

[25] S. Chen, N. Hirota, M. Okuda, M. Takeguchi, H. Kobayashi, N. Hanagata, T. Ikoma, Microstructures and rheological properties of tilapia fish-scale collagen hydrogels with aligned fibrils fabricated under magnetic fields, Acta Biomater. 7 (2011) 644–652.

[26] D.I. Zeugolis, S.T. Khew, E.S.Y. Yew, A.K. Ekaputra, Y.W. Tong, L.-Y.L. Yung, D.W. Hutmacher, C. Sheppard, M. Raghunath, Electro-spinning of pure collagen nano-fibres – Just an expensive way to make gelatin?, Biomaterials. 29 (2008) 2293–2305.

[27] R. Parenteau-Bareil, R. Gauvin, F. Berthod, Collagen-Based Biomaterials for Tissue Engineering Applications, Materials. 3 (2010) 1863–1887.

[28] X. Zhang, M. Ookawa, Y. Tan, K. Ura, S. Adachi, Y. Takagi, Biochemical characterisation and assessment of fibril-forming ability of collagens extracted from Bester sturgeon Huso huso × Acipenser ruthenus, Food Chem. 160 (2014) 305–312.

[29] M.T.I. Mredha, X. Zhang, T. Nonoyama, T. Nakajima, T. Kurokawa, Y. Takagi, J.P. Gong, Swim bladder collagen forms hydrogel with macroscopic superstructure by diffusion induced fast gelation, J. Mater. Chem. B. 3 (2015) 7658–7666.

[30] M.T.I. Mredha, N. Kitamura, T. Nonoyama, S. Wada, K. Goto, X. Zhang, T. Nakajima, T. Kurokawa, Y. Takagi, K. Yasuda, J.P. Gong, Anisotropic tough double network hydrogel from fish collagen and its spontaneous *in vivo* bonding to bone, Biomaterials. 132 (2017) 85–95.

[31] H.G. Sundararaghavan, G.A. Monteiro, N.A. Lapin, Y.J. Chabal, J.R. Miksan, D.I. Shreiber, Genipin-induced changes in collagen gels: Correlation of mechanical properties to fluorescence, J. Biomed. Mater. Res. Part A. 87A (2008) 308–320.

[32] G.R. Beck, E.C. Sullivan, E. Moran, B. Zerler, Relationship between alkaline phosphatase levels, osteopontin expression, and mineralization in differentiating MC3T3-E1 osteoblasts, J. Cell. Biochem. 68 (1998) 269–280.

[33] M.J. Dalby, M.O. Riehle, S.J. Yarwood, C.D.W. Wilkinson, A.S.G. Curtis, Nucleus alignment and cell signaling in fibroblasts: Response to a micro-grooved topography, Exp. Cell Res. 284 (2003) 274–282.

[34] A.J. Engler, S. Sen, H.L. Sweeney, D.E. Discher, Matrix Elasticity Directs Stem Cell Lineage Specification, Cell. 126 (2006) 677–689.

[35] R. Tatsumi, S.M. Sheehan, H. Iwasaki, A. Hattori, R.E. Allen, Mechanical Stretch Induces Activation of Skeletal Muscle Satellite Cells in Vitro, Exp. Cell Res. 267 (2001) 107–114.

[36] B.K.K. Teo, S.T. Wong, C.K. Lim, T.Y.S. Kung, C.H. Yap, Y. Ramagopal, L.H. Romer, E.K.F. Yim, Nanotopography Modulates Mechanotransduction of Stem Cells and Induces Differentiation through Focal Adhesion Kinase, ACS Nano. 7 (2013) 4785–4798.

[37] Y. Yang, K. Wang, X. Gu, K.W. Leong, Biophysical Regulation of Cell Behavior—Cross Talk between Substrate Stiffness and Nanotopography, Engineering. 3 (2017) 36–54.

[38] W.A. Loesberg, J. te Riet, F.C.M.J.M. van Delft, P. Schön, C.G. Figdor, S. Speller, J.J.W.A. van

Loon, X.F. Walboomers, J.A. Jansen, The threshold at which substrate nanogroove dimensions may influence fibroblast alignment and adhesion, Biomaterials. 28 (2007) 3944–3951.

[39] A.P. Gilmore, L.H. Romer, Inhibition of focal adhesion kinase (FAK) signaling in focal adhesions decreases cell motility and proliferation., Mol. Biol. Cell. 7 (1996) 1209–1224.

[40] A.P. Gilmore, Anoikis, Cell Death Differ. 12 (2005) 1473–1477.

[41] R.K. Assoian, M.A. Schwartz, Coordinate signaling by integrins and receptor tyrosine kinases in the regulation of G1 phase cell-cycle progression, Curr. Opin. Genet. Dev. 11 (2001) 48–53.

[42] R. Matsumoto, T. Uemura, Z. Xu, I. Yamaguchi, T. Ikoma, J. Tanaka, Rapid oriented fibril formation of fish scale collagen facilitates early osteoblastic differentiation of human mesenchymal stem cells, J. Biomed. Mater. Res. Part A. 103 (2015) 2531–2539.

[43] K. Sisson, C. Zhang, M.C. Farach-Carson, D.B. Chase, J.F. Rabolt, Fiber diameters control osteoblastic cell migration and differentiation in electrospun gelatin, J. Biomed. Mater. Res. Part A. 94A (2010) 1312–1320.

[44] K. Hoshi, S. Kemmotsu, Y. Takeuchi, N. Amizuka, H. Ozawa, The Primary Calcification in Bones Follows Removal of Decorin and Fusion of Collagen Fibrils, J. Bone Miner. Res. 14 (1999) 273–280.

[45] J. Richards, D. Pasco, J. Yang, R. Guzman, S. Nandi, Comparison of the growth of normal and neoplastic mouse mammary cells on plastic, on collagen gels and in collagen gels, Exp. Cell Res. 146 (1983) 1–14.

[46] W.-M. Zhang, J. armo Käpylä, J. S. Puranen, C. G. Knight, C.-F. Tiger, O. T. Pentikäinen, M. S. Johnson, R. W. Farndale, J. Heino, D. Gullberg, α11β1 integrin recognizes the GFOGER sequence in interstitial collgens, J. Biol. Chem. 278 (2003) 7270–7277.

[47] X. Zhang, Studies on the properties of sturgeon collagen as potential biomaterials, Doctoral thesis, Hokkaido University (2015) pp.123.

[48] X. Zhang, N. Azuma, S. Hagihara, S. Adachi, K. Ura, Y. Takagi, Characterization of type I and II procollagen α1chain in Amur sturgeon (*Acipenser schrenckii*) and comparison of their gene expression, Gene 579 (2016) 8–16.

Figure captions

Fig. 1. Scanning electron microscope images of the bottom surfaces of the cell culture wells coated with collagen. In (A), the well was coated with a porcine skin type I atelocollagen solution (0.03%) and washed with 0.1 M Na-phosphate buffer (PB, pH 7.6) (porcine collagen-molecule-coated well). In (B), the well was coated with a Bester sturgeon swim bladder type I atelocollagen (SBC) solution (0.03%) and washed with PB (SBC-molecule-coated well). In (C), the well was coated with an SBC solution (1.0%) and fibril formation and cross-linking were simultaneously induced by the PB containing 1 mM genipin (fine-fibril-coated well). In (D), the well was coated with an SBC solution (1.0%), fibril formation was induced by a PB, and cross-linking was induced sequentially by the PB containing 1 mM genipin (coarse-fibril-coated well). Scale bars, 5 μm.

Fig. 2. Distribution of the collagen fibril diameter of the fine-fibril-coated well (white columns) and the coarse-fibril-coated well (black columns).

Fig. 3. Phase contrast microscope images of MC3T3-E1 cells cultured for 1 hour (A, D, G, J), one day (B, E, H, K), and seven days (C, F, I, L) on the porcine collagen molecule-coated well (A-C), the SBC molecule-coated well (D-F), the fine-fibril-coated well (G-I), and the coarse-fibril-coated well (J-L). Scale bars, 75 μm.

Fig. 4. Phase contrast microscope images of ATDC5 cells cultured for 3 hours (A, D, G, J), one day (B, E, H, K), and seven days (C, F, I, L) on the porcine collagen molecule-coated well (A-C), the SBC molecule-coated well (D-F), the fine-fibril-coated well (G-I), and the coarse-fibril-coated well (J-L). Scale bars, 75 μm.

Fig. 5. Proliferation of MC3T3-E1 cells (A) and ATDC5 cells (B) on the porcine collagen molecule-coated well (white column), the SBC molecule-coated well (black column), on the fine-fibril-coated well (hatched column), and the coarse-fibril-coated well (dotted column). Columns and bars indicate means and standard errors. Different letters in the same day indicate significant differences ($p < 0.05$ by Tukey-Kramer test).

Fig. 6. ALP activities of MC3T3-E1 cells cultured on the porcine collagen molecule-coated well (white column), the SBC molecule-coated well (black column), the fine-fibril-coated well (hatched column), and the coarse-fibril-coated well (dotted column). Columns and bars indicate means and standard errors. Different letters in the same day indicate significant differences ($p < 0.01$ by Tukey-Kramer test).

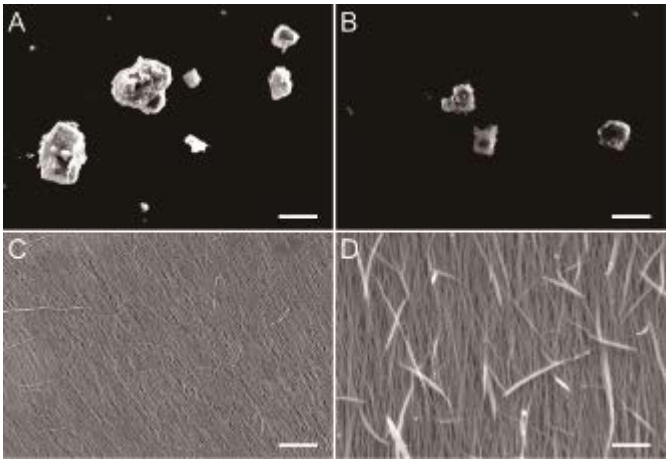
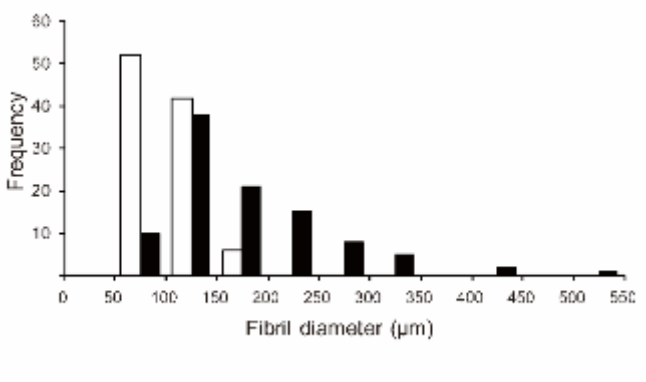


Fig. 1


Frequency
60
50
40
30
20
10
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50
100
150
200
250
300
350
400
450
500
550
Fibril diameter (µm)

Fig. 2

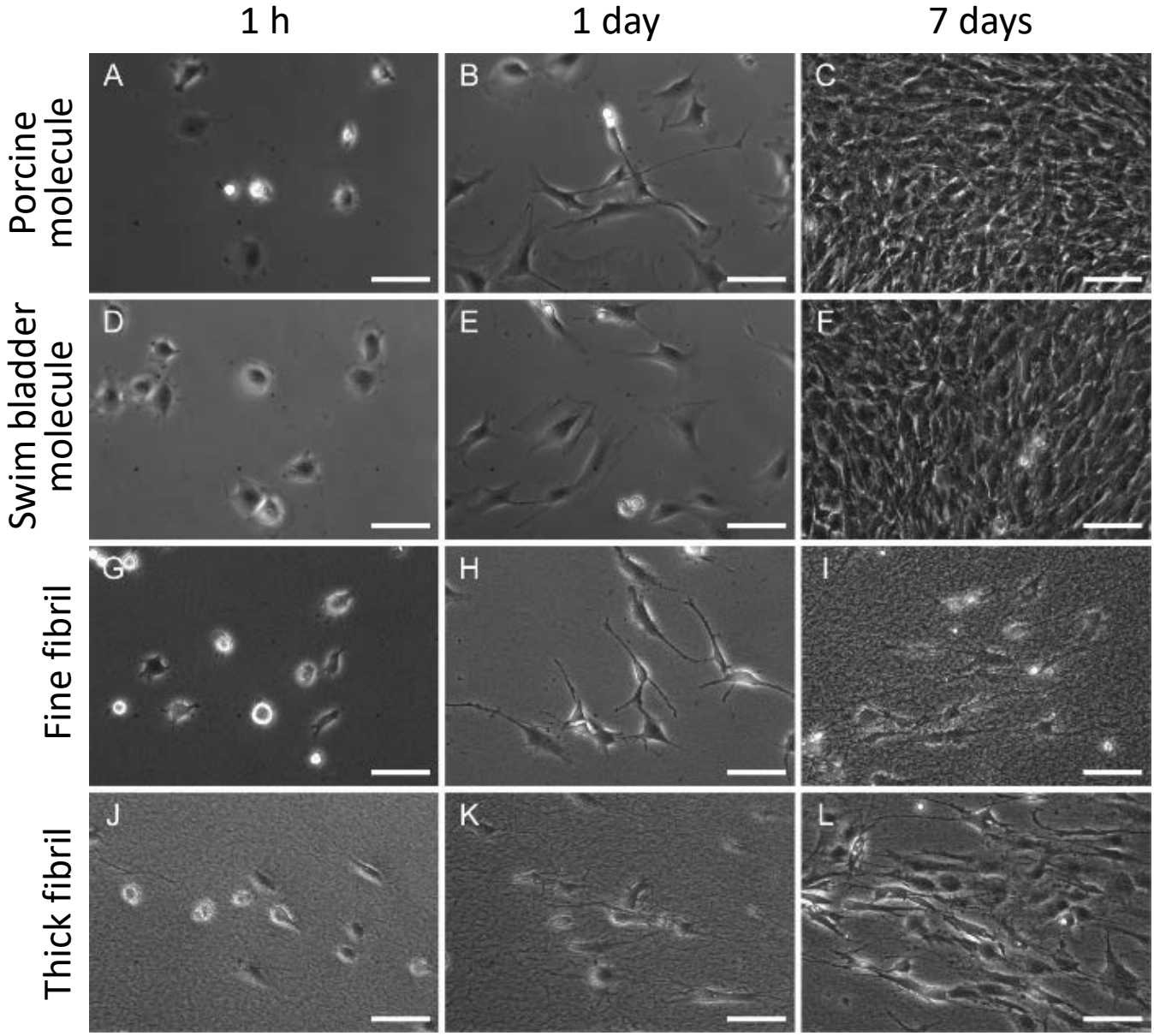
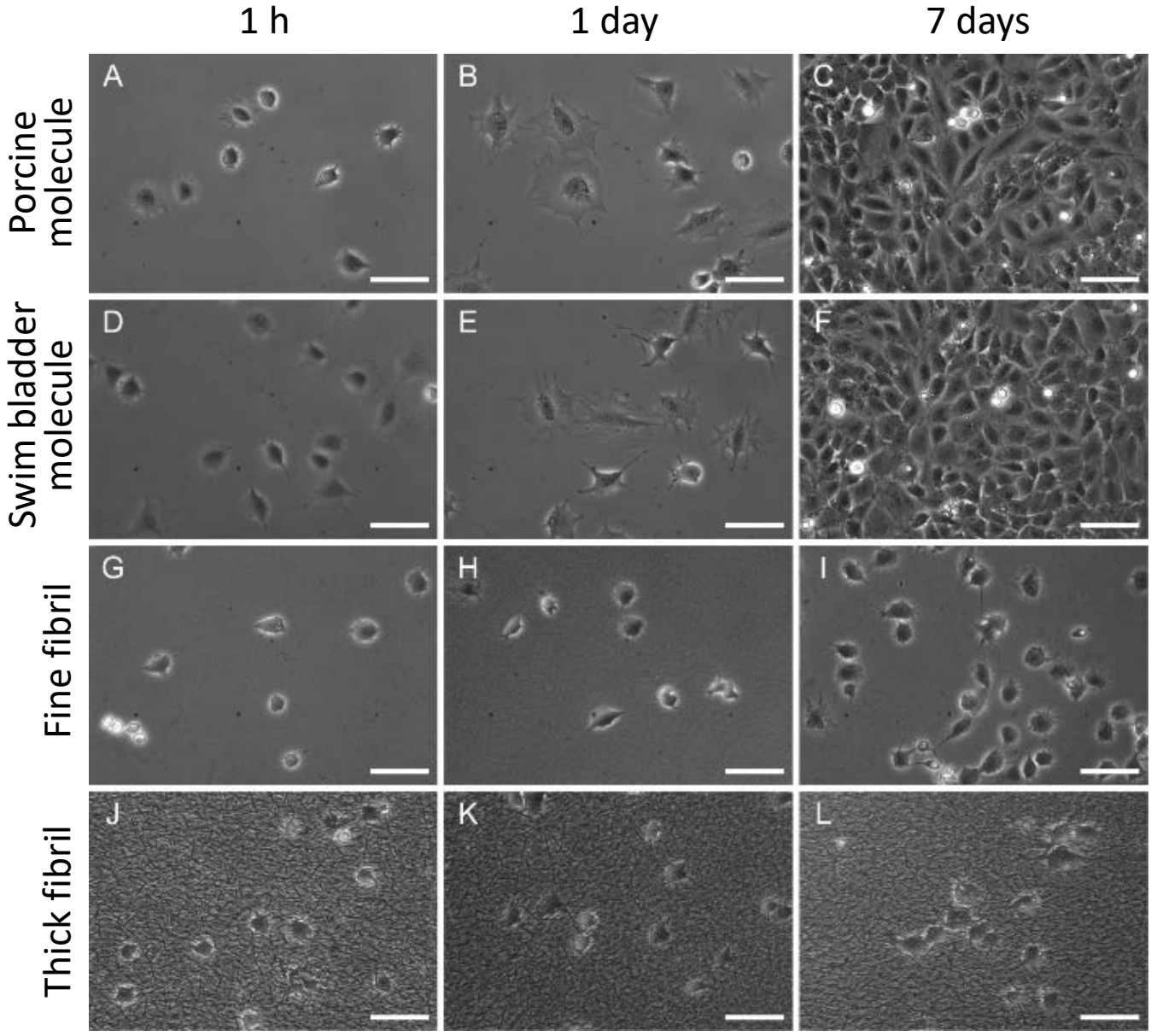

1 h
1 day
7 days
Porcine molecule
Swim bladder molecule
Fine fibril
Thick fibril
A
B
C
D
E
F
G
H
I
J
K
L

Fig. 3


1 h
1 day
7 days
Porcine molecule
Swim bladder molecule
Fine fibril
Thick fibril
A
B
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D
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K
L

Fig. 4

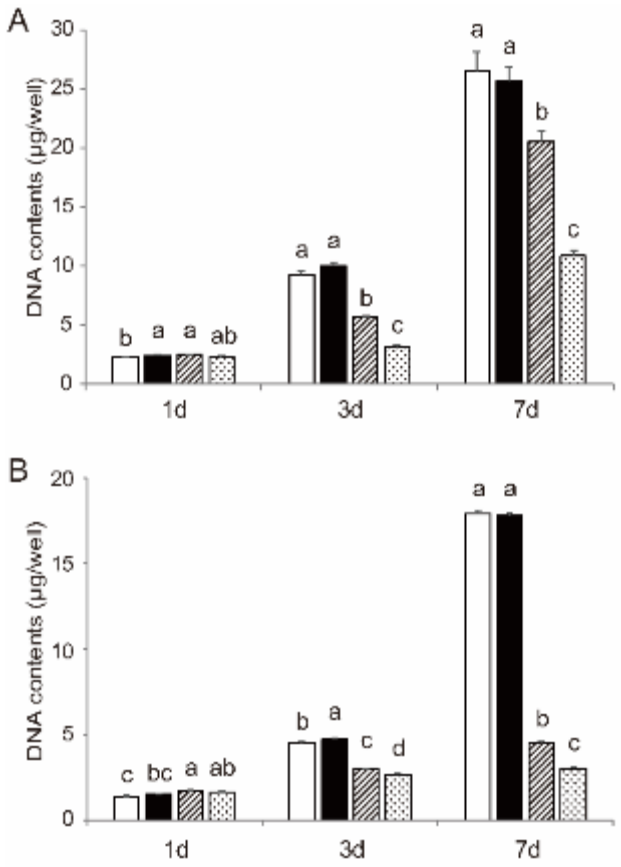

A
DNA contents (μg/well)
30
25
20
15
10
5
0
b a a ab
a a b c
a a b c
1d
3d
7d
B
DNA contents (μg/well)
20
15
10
5
0
c bc a ab
b a c d
a a b c
1d
3d
7d

Fig. 5

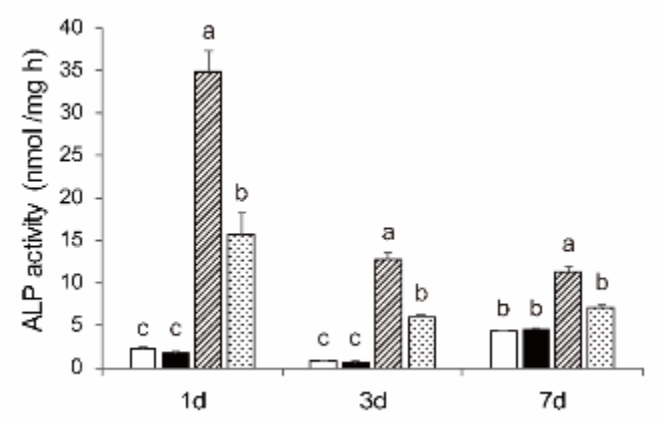
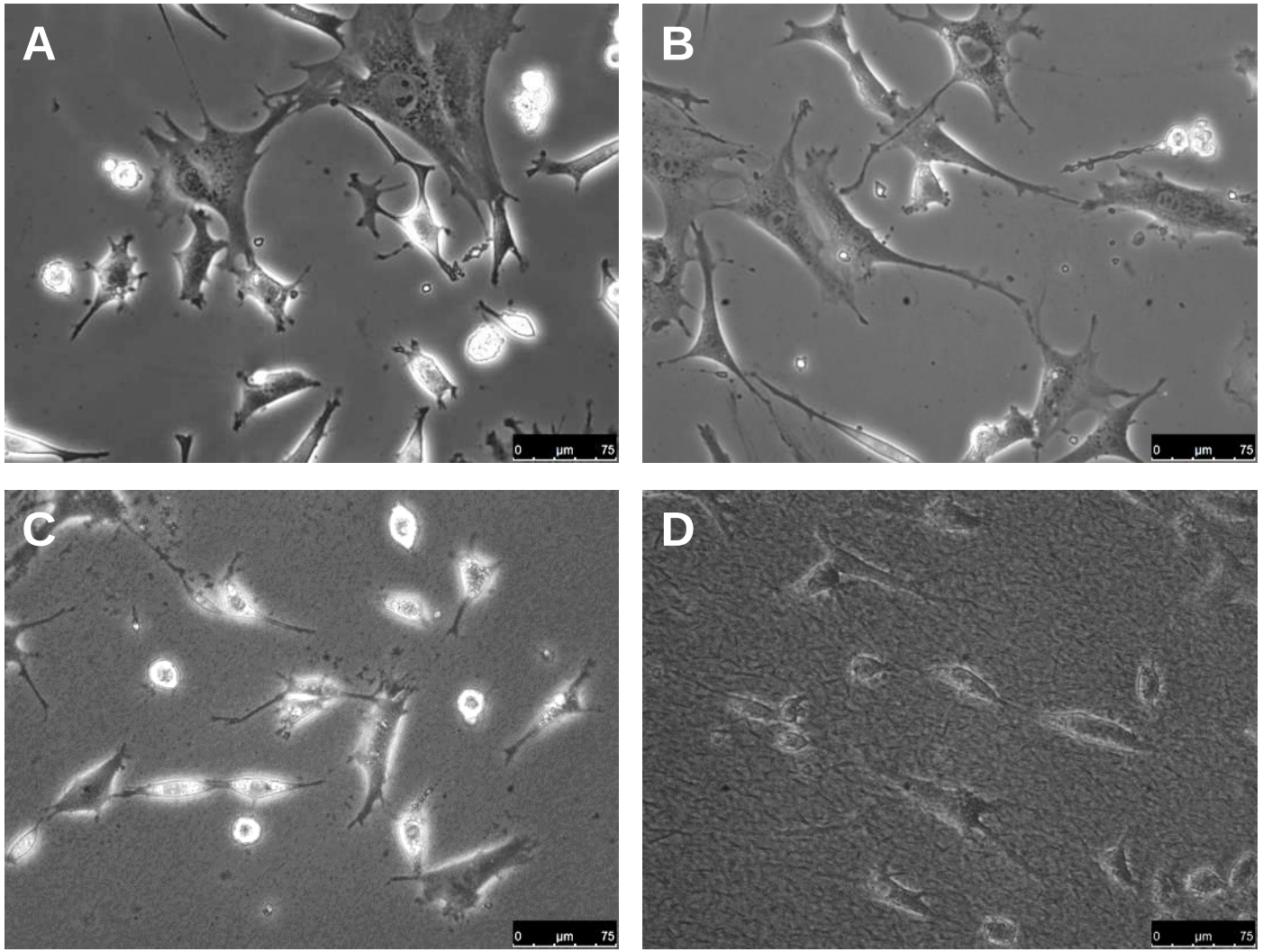
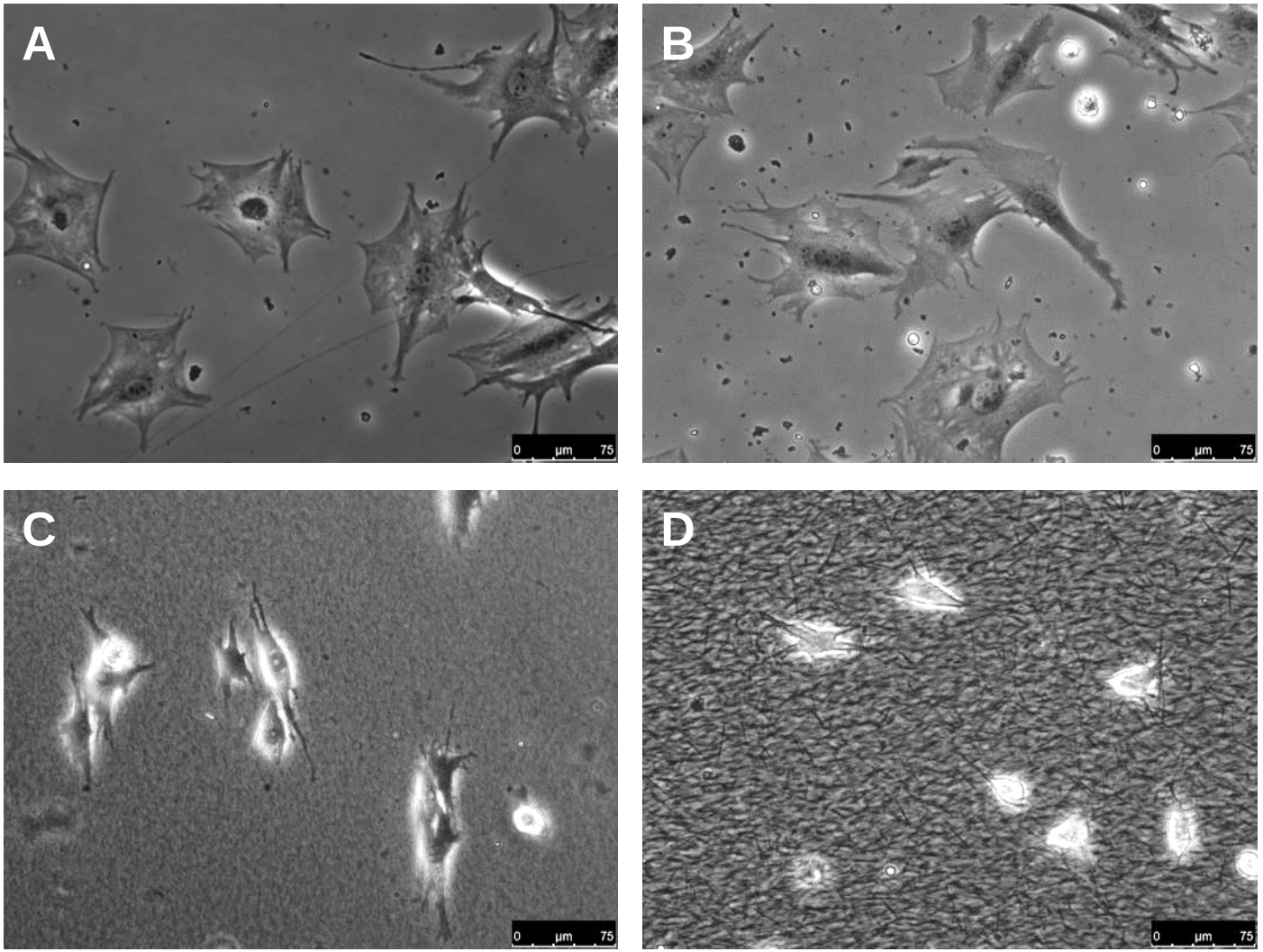

ALP activity (nmol /mg h)
40
35
30
25
20
15
10
5
0
a
b
a
b
a
b
c c
c c
b b
1d
3d
7d

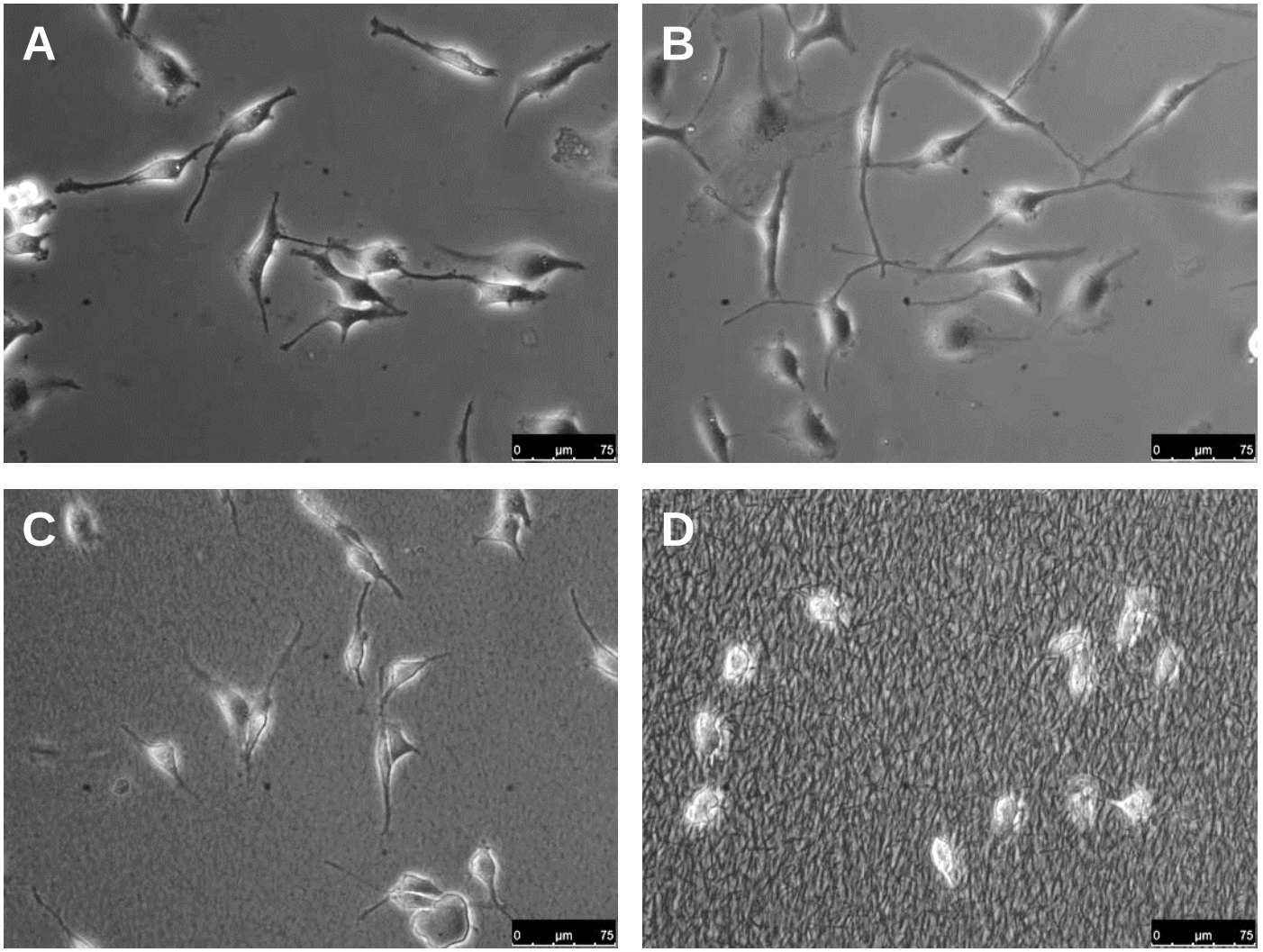
Fig. 6



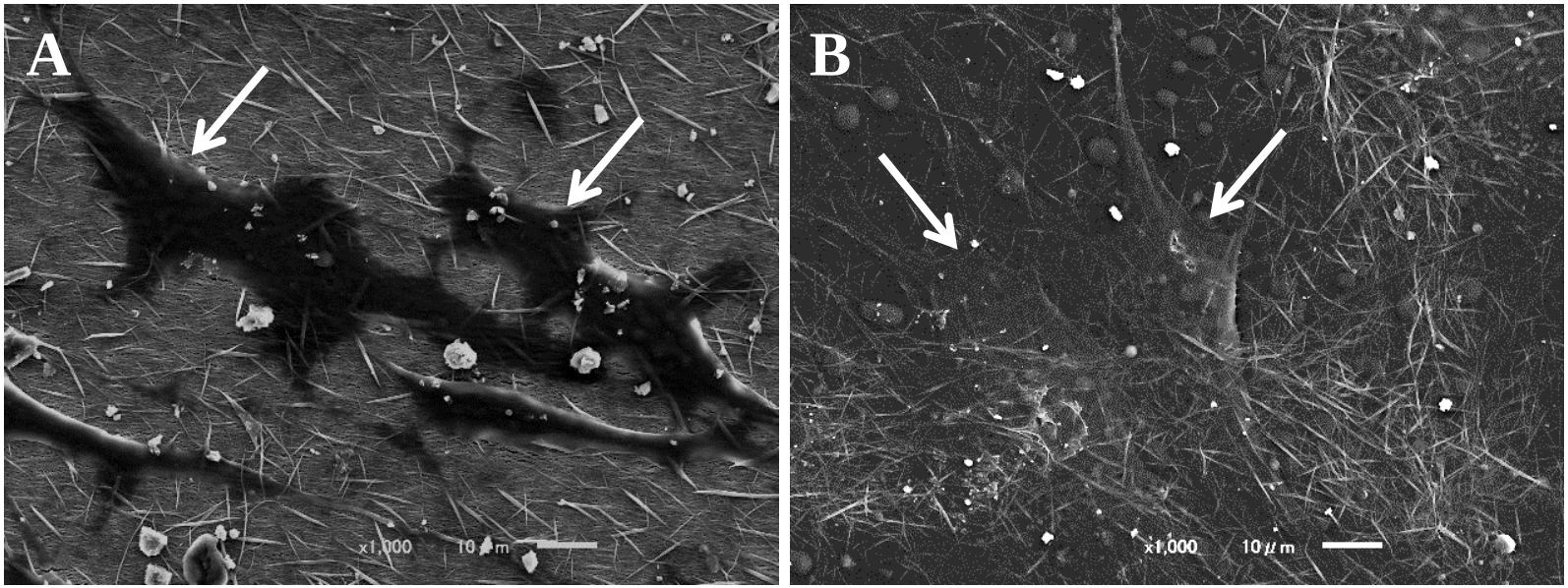
Supplementary Fig. 1. Phase contrast microscopy images of 3T3-L1 cells cultured for 1 day on the porcine collagen molecule-coated well (A), the SBC molecule-coated well (B), the fine-fibril-coated well (C), and the coarse-fibril-coated well (D).



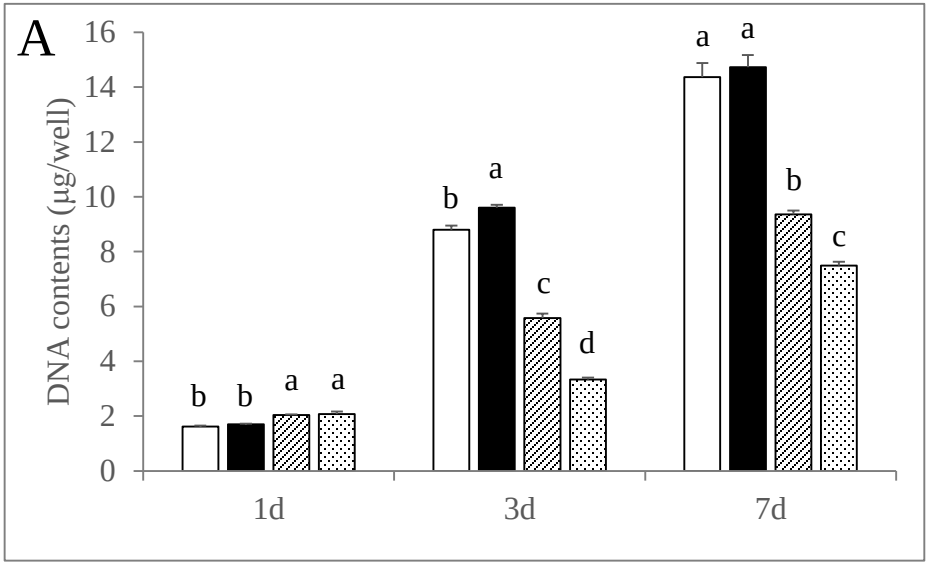
Supplementary Fig. 2. Phase contrast microscopy images of C2C12 cells cultured for 1 day on the porcine collagen molecule-coated well (A), the SBC molecule-coated well (B), the fine-fibril-coated well (C), and the coarse-fibril-coated well (D).

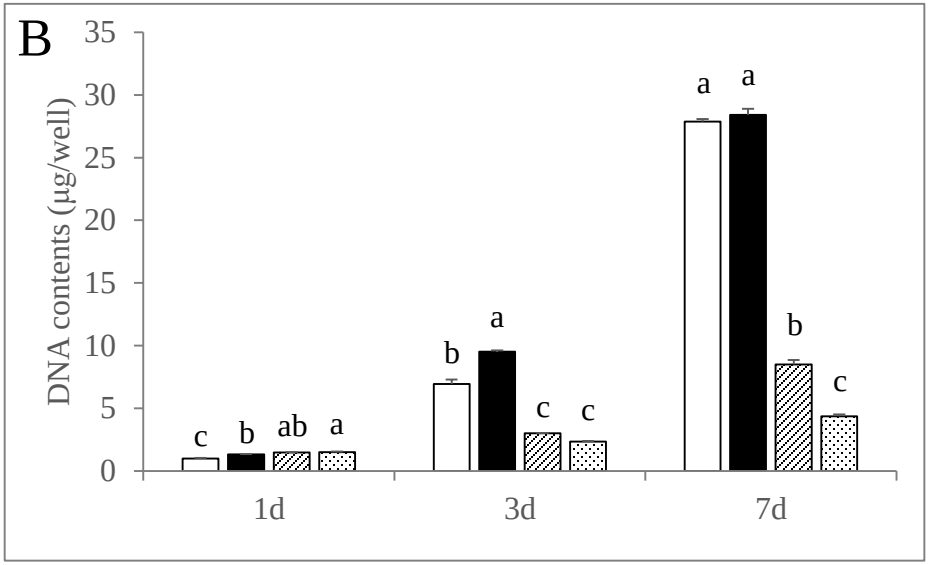


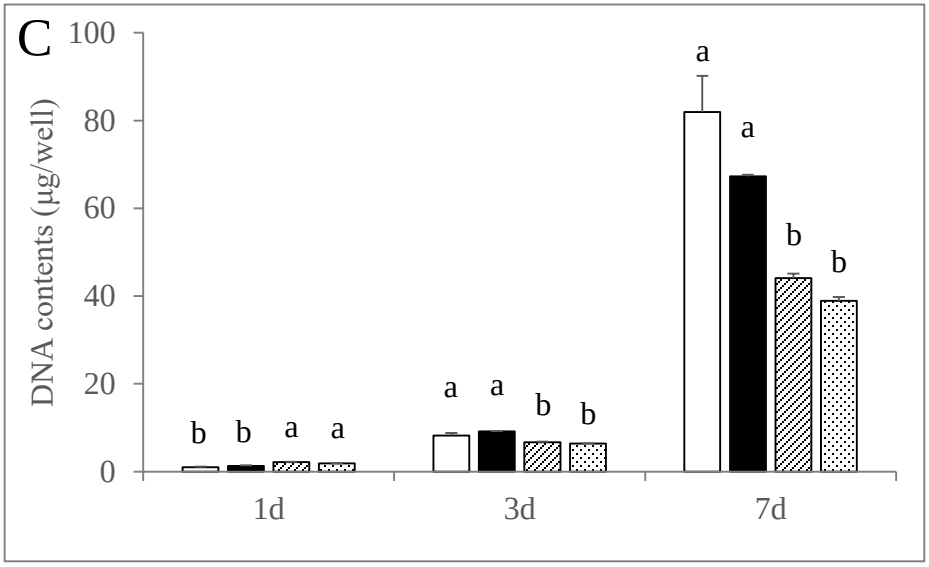
Supplementary Fig. 3. Phase contrast microscopy images of L929 cells cultured for 1 day on the porcine collagen molecule-coated well (A), the SBC molecule-coated well (B), the fine-fibril-coated well (C), and the coarse-fibril-coated well (D).



Supplementary Fig. 4. Scanning electron microscope images of the bottom surfaces of the cell culture wells coated with collagens fibrils. A 200 μl aliquot of the atelocollagen solution (1%) was poured into each well of the 24-well cell-culture plate, incubated for 30 minutes, and removed from each well. The fibril formation was induced by introducing a 0.1 M Na-phosphate buffer (PB, pH 7.6) into the wells for 24 hours. MC3T3-E1 cells were cultured after crosslinking with the PB containing genipin for subsequent 24 hours (A) or without cross linking (B). After culture for 1 day, the morphology of the crosslinked fibrils were the same as that before the culture (see Fig. 1D in the manuscript). Without the genipin crosslinking, randomly aligned collagen fibrils were observed. Arrows indicate cells.







Supplementary Fig. 5. Proliferation of 3T3-L1 (A), C2C12 (B), and L929 cells (C) on the porcine collagen molecule-coated well (white column), the SBC molecule-coated well (black column), the fine-fibril-coated well (hatched column), and the coarse-fibril-coated well (dotted column). Columns and bars indicate means and standard errors. Different letters in the same day indicate significant differences ($p < 0.05$ by Tukey-Kramer test).

Highlights

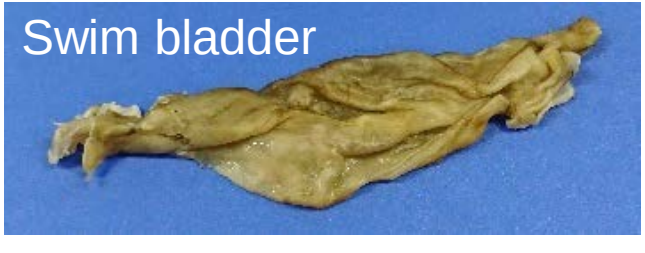
- Atelocollagen from the swim bladder of sturgeon forms oriented fibrils
- Two types of fibrils were coated on cell culture dishes
- Thick collagen fibrils induced cells to align in the same direction
- Cellular proliferation was inhibited on both thin and thick fibrils
- Both fibrils stimulated the early differentiation of MC3T3-E1 cells

Graphical abstract

Processing

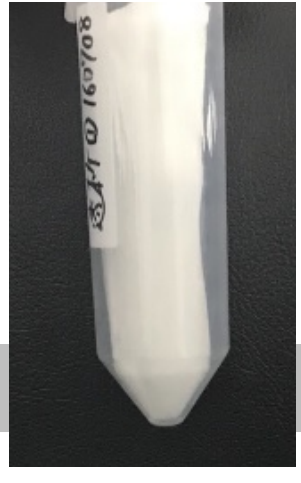
Fillet, Caviar

By-products



Extraction

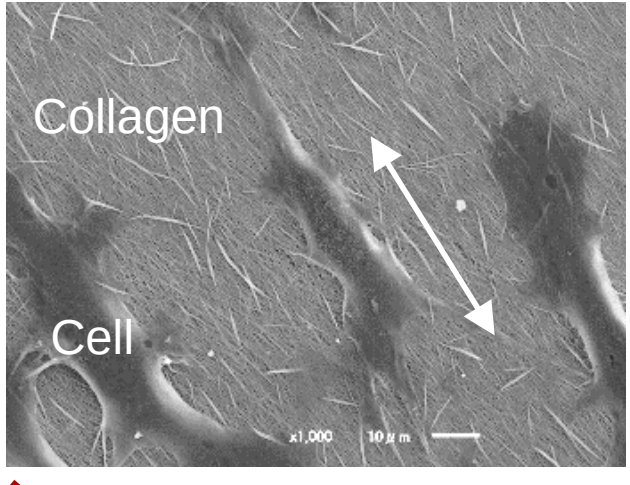
Purified collagen



Coating

Highly-oriented collagen fibrils on the dish

Cells aligned along the fibrils



Cell proliferation

ALP activity (MC3T3-E1)