Engineered whole cut meat-like tissue by the assembly of cell fibers using tendon-gel integrated bioprinting

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With the current interest in cultured meat, mammalian cell-based meat has mostly been unstructured. There is thus still a high demand for artificial steak-like meat. We demonstrate in vitro construction of engineered steak-like tissue assembled of three types of bovine cell fibers (muscle, fat, and vessel). Because actual meat is an aligned assembly of the fibers connected to the tendon for the actions of contraction and relaxation, tendon-gel integrated bioprinting was developed to construct tendon-like gels. In this study, a total of 72 fibers comprising 42 muscles, 28 adipose tissues, and 2 blood capillaries were constructed by tendon-gel integrated bioprinting and manually assembled to fabricate steak-like meat with a diameter of 5 mm and a length of 10 mm inspired by a meat cut. The developed tendon-gel integrated bioprinting here could be a promising technology for the fabrication of the desired types of steak-like cultured meats.

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ver the past decade, cultured meat has drawn tremendous attention from the standpoints of ethics, economics, the environment, and public health, although it is still under debate. More recently, meat analogs that taste like meat but are based on plant proteins have been released commercially. Although challenges remain unlike with meat analogs, cultured meat is highly sought after due to the possibility of imitating real meat through the manipulation of flavor, muscle/adipose cells’ ratio, and texture.

Bovine cells for cultured meat can currently be secured by two approaches. One is following the obtention of edible muscle tissues from cattle, their cells are separated into each type such as muscle satellite cells, adult stem cells, and multipotent stem cells, which are then cultured to increase the number of cells. The other is from transforming somatic cells into induced pluripotent stem cells (iPSCs) and differentiating them into each cell type. Primary cultured stem cells, particularly muscle satellite cells, maintain their differentiation capability within 10 passages and thus have a limited number of divisions. But, they should still be safe and acceptable for consumption.

Since Post and coworkers unveiled bovine cell fiber-based hamburger, various types of cultured meat have been demonstrated. However, cultured steak with a composition and a structure similar to real steak, comprising mostly adipose cells and aligned fibers, has not yet been achieved. Although challenges remain unlike with meat analogs, cultured meat through the manipulation of muscle fibers alignment and maturation, we fabricated tendon gels by TIP to enable their consecutive connection with the muscle cell fibers, inducing the formation of aligned matured muscle fibers. In this study, a total of 72 fibers comprising 42 muscle, 28 adipose, and 2 blood capillaries were constructed by TIP. They were subsequently assembled to fabricate steak-like meat with a diameter of 5 mm and a length of 10 mm inspired by the histological images of an actual Wagyu beef steak. TIP is expected to become a powerful approach for constructing engineered steak-like meat with desired location, component ratio, and amount of the three types of fibers.

Results

Verification of the differentiation conditions for the extracted bSCs and bADSCs. The bSCs were extracted from the massecut muscle of 27-month-old Japanese black cows obtained from a slaughterhouse using a method modified from a previously reported one. The crude cell fraction separated from the beef meat by collagenase treatment was cultured until passage 3 (P3) for cell sorting. The CD31+ , CD45− , CD56+ , and CD29+ cells were isolated by fluorescence-activated cell sorting (Supplementary Fig. 1), in which Pax7+ bSCs were around 80%. 2D culture of the isolated bSCs was performed to evaluate their proliferation and differentiation potentials into muscle cells even after prolonged passaging. After seeding, the bSC passage was incremented after each cell detachment by trypsinization every two days. The proliferation medium contained not only fetal bovine serum (FBS) but also a p38 inhibitor to maintain the differentiation potential of the proliferating bSCs. The number of seeded bSCs doubled approximately once per day until P8, and about once every two days thereafter (Fig. 2a). The differentiation was induced after two days of seeding by switching the basic medium for a differentiation media containing 2% horse serum (HS), which is a well-known differentiation-induction method for muscle cells. The cells were immunostained with the antibody of myosin II heavy chain (MHC) after five days of differentiation induction. We quantified the differentiation capacity regarding the passage number of the seeded bSCs by calculating the ratio of DAPI fluorescence intensity between MHC+ and MHC− cells from fluorescence images (Supplementary Fig. 2). The bSCs from P3 to P7 expressed a comparable differentiation level, but the differentiation capability of bSCs above P8 significantly decreased (Figs. 2b and 2c). Therefore, we conducted experiments using cells prior to P8.

Next, 3D culture with collagen microfibers (CMF)/fibrin gel was performed for assessing the adipogenic differentiation potential of the bADSCs in a variety of media conditions since it is known that the adipogenesis of adipose-derived stem cells (ADSCs) in 3D culture is higher than in 2D culture and that the differentiation-factor efficiency relies on species. Conventional human adipogenic factors like insulin, rosiglitazone, or troglitazone were thus first found with limited lipogenesis (Supplementary Fig. 3), leading to the direct addition of free fatty acids (pristanic acid, phytic acid, erucic acid, elaidic acid, oleic acid, palmitoleic acid, and myristoleic acid) to the culture medium following previous published methods. The different combinations of the seven aforementioned free fatty acids were also compared and the results showed a higher lipogenesis by lipid storage into the cytoplasmic vesicles of the bovine preadipocytes for the medium containing all the seven free fatty acids (Fig. 2d). To further increase the lipogenesis until reaching a matured bovine adipocyte state, the transforming growth factor (TGF) type 1 receptor activin-like kinase 5 inhibitor (ALK5I) effect was evaluated because this factor is an inhibitor of
the TGF-β receptor ALK5 and TGF-β family ligands, contained in the 10% FBS of the culture medium, which is known to inhibit both adipogenesis and adipocyte hypertrophy\(^\text{31}\). The TGF-β family also includes myostatin, which is expressed by the myocytes to impair adipogenesis\(^\text{32}\). In the context of future coculture between bovine myoblasts and adipocytes, ALK5i appeared relevant for further inducing the adipogenic potential of the culture medium containing the seven free fatty acids. Several concentrations were thus assessed from 1 to 10 µM. The results showed a tendency to a higher lipogenesis with 5 µM ALK5i (Fig. 2e and Supplementary Fig. 5a). The lipogenesis of the bADSCs then increased progressively between 3 and 14 days of differentiation (Fig. 2f and Supplementary Fig. 5b).

In addition to lipogenesis, further investigations concerning the two adipogenic markers PPAR\(^\gamma\) and FABP4 were conducted to evaluate the adipogenesis. The immunostaining of PPAR\(^\gamma\), one of the most important transcription factors for fat cell differentiation, showed a slight expression inside the bADSCs after three days of differentiation, which was specifically found in the nuclei due to their role as an early transcription factor, inducing the other adipogenic maturation genes\(^\text{33}\) (Supplementary Fig. 6). This location in the nuclei is less observed after 14 days of differentiation, implying a more matured state of the bADSCs (Fig. 2g and Supplementary Fig. 6). Concerning FABP4, a late specific adipogenic marker necessary for trafficking fatty acids to the membrane for efflux\(^\text{34}\), its expression location was observed in the cytoplasm, increasing following the differentiation duration with a particularly high expression observed in the unilocular mature adipocytes (Fig. 2g and Supplementary Fig. 6). The increase of both early and late marker expressions was confirmed by the RNA (qPCR) analysis, which highlighted the significant linear-increasing expression profile of the PPAR\(^\gamma\) marker, followed by the FABP4 to a higher extent testifying the mature state of the bADSCs-derived adipocytes (Fig. 2h).

Recently, ADSCs have been considered to be a useful cell source for angiogenesis in tissue engineering, but unlike human ADSCs, there are no reports on endothelial differentiation of bADSCs\(^\text{35,36}\). Knowing that they can lose their differentiation potentials during ADSCs culture expansion\(^\text{37}\), bADSCs were thus used at P1 to evaluate their endothelial differentiation in different conditions. Horse serum (HS) was surprisingly found to be a significant inducer of the CD31 expression, an endothelial cell marker (4 and 15 times more for 1 and 10% HS, compared with the 10% FBS condition), independently of the medium used, DMEM or F12K (Fig. 2i–j and Supplementary Fig. 7). The human serum also provided an enhanced endothelial differentiation, compared with the FBS condition, but was impaired by the low cell proliferation observed (Supplementary Fig. 7). In addition to CD31 expression, the tubulogenesis was confirmed by culturing the seven-day differentiated cells on Matrigel in media containing 10% HS.
(Supplementary Fig. 8). The DMEM + 10% HS was then used for the endothelial differentiation from bADSCs in this study.

**Bovine muscle fiber fabrication by supporting bath-assisted 3D printing.** To organize the isolated bSCs into a cell fiber, we utilized a supporting bath-assisted 3D printing (SBP) consisting of a bioink dispensed inside a supporting bath usually composed of a hydrogel slurry that ensures the printed-structure stability in the z axis. Several studies have also demonstrated its promise in cell printing for its high-shape fidelity even on complex or soft structures, and for its stable printing during prolonged operation\(^\text{20,21,23,24}\). We selected gelatin and gellan gum as supporting bath materials, due to their edible, removable, and cell-compatible properties. Gelatin is a gel at room temperature (RT) and a liquid at 37 °C, therefore, it is easy to remove after printing by incubation at 37 °C\(^\text{19}\). Gellan gum hydrogel is also known to dissolve in 50 mM Tris-HCl buffer at pH 7.4 and at 37 °C\(^\text{38}\). Hydrogel slurry was fabricated by homogenizing bulk hydrogel of gellan gum and gelatin, in which the average particle sizes are 44 μm and 70 μm, respectively, and their thixotropy behavior was confirmed (Supplementary Fig. 9).
First, we tried to print the bioink containing bSCs, fibrinogen, and Matrigel solution in the culture medium into the supporting bath mixed with granular particles of gelatin (G-Gel) or gelatin gum (G-GG) and thrombin for the fabrication of a fibrous muscle fiber mimicking the structure of muscle fiber in steak (Supplementary Movies 1 and 2). With the confirmation of the gel formation followed by the removal of supporting baths, high cell viability was observed for nine days after printing both in the G-Gel and G-GG by live/dead staining (Figs. 3a, 3b and Supplementary Fig. 10).

When the printed cell fiber was cultured in suspension, the fiber collapsed to a globular form (Fig. 3c). Studies related to muscle tissue engineering have implied that an anchoring structure enables the 3D muscle tissues to not only maintain their initial shape but also to improve the cell alignment, fusion, and differentiation against the muscle fiber’s contraction. We placed a printed cell fiber onto a silicone rubber and anchored it with needles fastening both ends to withstand cell contractions (Fig. 3d, left). With the needle-fixed culture, the cell fibers printed inside G-GG and G-Gel retained the fibrous structure, but the diameter shrank by around 60% in G-GG and 80% in G-Gel at day 9 of culture (Fig. 3d, right). It would be reasonable to suppose that the size decrease was caused by the alignment and fusion of bSCs along with the enzymatic decomposition of fibrin gel by the proteases secreted from the cells. We also took immunofluorescence images inside the cell fibers to examine the cellular behavior w/ and w/o needle anchoring on day 3 of differentiation. To quantify the improvement in terms of muscle maturation, the cell alignment, i.e., the angle setting for the straight line between needles, was measured from the immunofluorescence images (Fig. 3e, left). The results showed that the cells in the cell fiber of the suspension culture were randomly oriented, regardless of the type of supporting baths, while in the needle-fixed culture the cells in the cell fiber printed inside G-Gel were anisotropically oriented compared with those of G-GG (Fig. 3e, right). We postulated that the difference in the degree of alignment between G-GG and G-Gel arises from the hindrance of cell behaviors by some residual substances that might exist inside or on the printed cell fibers. This substance was found to be the residual G-GG in the cell fiber (Supplementary Fig. 11), which may not be degraded or dissolved, limiting the cells in their ECM remodeling required to migrate and fuse with the other cells. On the other hand, G-Gel is easily dissolved at 37 °C and may be degraded by proteases, enabling an active cell behavior, despite the possible residues that might remain in the printed cell fiber. Printing bSCs inside G-Gel and anchoring them are the essential steps for the fabrication of the muscle cell fiber, but the anchoring method may not be appropriate for the scale-up. Therefore, we developed a modified SBP to include a part that can be simultaneously anchored by the printed cell fiber.

Fabrication of muscle, fat and vascular cell fibers by TIP. The important feature in the modified SBP, which we have named tendon-gel-integrated bioprinting (TIP), is the introduction of tendon gels to anchor the printed cell fibers for culture. Figure 4a illustrates the process of the TIP in which the printing bath is divided into three parts: the bottom tendon gel, the supporting bath, and the upper tendon gel. G-Gel is used as a supporting bath as described in the above section and the volume of tendon-gels is filled with 4 wt% collagen nanofiber solution (CNFs) which has a reversible sol-gel transition from 4°C to 37°C (Supplementary Fig. 12). To separate the layers and maintain the structure we fabricated polydimethylsiloxane (PDMS) wells (Supplementary Fig. 13). After the bSC fiber gelation inside the PDMS well (Supplementary Movie 3), incubation for 2 h at 37°C induced the supporting bath and tendon gels to become a solution and a gel, respectively, and the PDMS well was then put in the culture medium.

On day 3, we could confirm that the printed cell fiber maintained its fibrous shape and kept its connection with the two tendon gels, as seen in the phase contrast and the H&E staining images (Figs. 4b and 4c). The cell viability of the bSC fiber by TIP was confirmed up to day 9 (Supplementary Fig. 14). It also showed a high alignment of cells on day 3 of differentiation, which seemed comparable with the needle-fixed culture (Figs. 3e, 4d, and 4e and Supplementary Movie 4). MHC expression of the TIP-derived bSC fiber was relatively lower than in the needle-fixed culture (Supplementary Fig. 15), but the mRNA level of MHC expression was upregulated by >1000-fold on day 3 of differentiation compared with the naive bSCs (Fig. 4f). Interestingly, sarcomere structures testifying a matured state for the muscle fibers were shown in some of the TIP-derived bSC fibers (Fig. 4g), but we could not show any needle-fixed culture cell fibers after 14 days of differentiation for the comparison. These results imply that the long-term culture in TIP is able to induce a higher muscle-maturation degree compared with the needle-fixed culture. Even though we did not investigate thoroughly here, it may have been caused by the bSC cell adhesion to the collagen gel at anchorage regions for the TIP whereas there is no cell adhesion in the needle-fixed culture (Supplementary Fig. 16). TIP is a promising method for muscle fiber fabrication, but it still has a problem which is the occasional bSC fiber detachment from the tendon gels, especially the bottom tendon gels, during the prolonged cultures due to its strong contraction. Increasing the concentration of the CNFs or using additional cross-linking will hopefully provide a solution to this problem. Moreover, double printing by the addition of...
another cell fiber by general TIP after rotating the PDMS well 180° following the fabrication of the first one, may be another way of solving the problem. When double printing is performed, the two printed cell fibers close to each other fused into one thicker-cell fiber (Supplementary Fig. 17).

Multiple printing for 25 bSC cell fiber fabrication in one large PDMS well was also performed (Fig. 4h and Supplementary Movie 5). We first aimed to be able to produce directly in one PDMS well a large tissue composed of various types of cell fibers, but we finally fabricated the muscle, fat, and vascular cell fibers individually in this study because each differentiation needed to be induced in a specific medium corresponding to each cell fiber based on the information discussed in the first section. The adipogenesis of the bADSCs-derived fat fiber by TIP was confirmed by the mRNA level and protein expression of PPARγ2 and FABP4 same as in 3D culture. Compared with naive bADSCs, PPARγ2 and FABP4 were upregulated by >6-fold and >40-fold respectively in their mRNA expression and >2-fold and >2-fold, respectively, in their protein level on day 14 of differentiation (Fig. 4i and j and Supplementary Fig. 18). Figure 4k, Supplementary Fig. 19, and Supplementary Movies 6–8 show the whole muscle, fat, and blood capillary cell fibers, respectively, independently fabricated by TIP. Even though each cell fiber was fabricated separately, we believe that if a differentiated media for culturing all three types of cell fibers at the same time is developed, the programmed printing of them in desired locations will be feasible.

The characteristics of DNA amount, compressive modulus, and water contents of the muscle and fat cell fibers by TIP were compared with the fibers extracted from a commercial beef (Supplementary Fig. 20). The DNA concentration in the TIP-derived muscle fiber did not change, depending on the culture day while it increased in TIP-derived fat fiber over that of the commercial meat on day 14 of differentiation, implying the proliferation and the significant change in the cell numbers in fat fibers during the culture and the differentiation after printing, which was not the case for the muscle fibers (Fig. 4i). Also, the DNA concentration in the TIP-derived muscle fibers

Fig. 3 The characterization of bSC tissue fabricated by SBP. a, b Optical (left), phase contrast (middle), and fluorescence (right, green: live cells and red: dead cells) images of the bSC tissues printed inside granular gellan gum (G-GG) (a) and granular gelatin (G-Gel) (b) followed by bath removal. Scale bars, 500 μm. c Shape change of bSC tissue fabricated by SBP inside G-Gel from the fibrous form right after printing and bath removal to globular form on day 6 of suspension culture. d Schematic (left), size change in accordance with culture day (middle), and phase-contrast images (right) of needle fixed culture of printed bSCs tissues. Error bars represent mean ± s.d. Scale bars, 500 μm. e 3D-fluorescence images (upper, red: actin and green: MHC) and cell alignment measurements (lower) of the bSC tissues printed inside G-GG and G-Gel and in suspension and needle-fixed cultures on day 3 of differentiation (after six days), respectively. Representative images from at least two independent experiments are shown. Scale bars, 200 μm. Source data are provided as a Source Data file.
**Fig. 4** Tendon-gel integrated bioprinting (TIP) for muscle, fat, and vascular tissue fabrication. 

**a** The schematic of TIP for cell printing. **b** Optical (upper) and phase-contrast (lower) images of the bSC tissue printed by TIP, keeping the fibrous structure on day 3. The images were taken after fixation. Scale bar, 1 mm. **c** The H&E-stained image of half of collagen gel (dotted black line)—fibrous bSC tissue (dotted red line) and a magnified image of the fibrous bSC tissue (right). Scale bars, 2 mm (left) and 50 µm (right). **d** 3D fluorescence image (left) and cell alignment measurement (right) of the TIP-derived bSC tissue stained with actin (red), MHC (green), and nucleus (blue) on day 3 of differentiation. Scale bar, 50 µm. **e** SEM images of TIP-derived bSC tissue on day 3 of differentiation. Scale bars, 10 µm and 100 µm (inset). **f** MHC mRNA expression levels of bSCs before printing and TIP-derived bSC tissue on day 3 of differentiation (n = 3 independent samples, pairwise t-test comparison). **g** Fluorescence image of TIP-derived bSCs tissue stained with actin (red), MHC (green), and nucleus (blue) on day 14 of differentiation. Scale bar, 50 µm. **h** The optical images of multiple tissue fabrication (25 ea.) by multiple printing. Black arrows indicate printed cell fibers. **i–j** mRNA levels (i) and protein expression levels (j) of TIP-derived fat tissues before printing and at day 14 of differentiation (at day 17 of total culture) (n = 3 independent samples, pairwise t-test comparison). **k** Whole fluorescence (left), optical (inset), and magnified (right) images of muscle (on day 4 of differentiation, green: MHC & blue: nucleus), fat (on day 14 of differentiation, red: lipid and blue: nucleus), and vascular (on day 7, magenta: CD31 and blue: nucleus) tissues fabricated by TIP. Scale bars, 1 mm (left) and 100 µm (right). **l–m** DNA amount per weight (light-gray bars: day 1, and dark-gray bars: day 6 in muscle fiber and day 17 in fat fiber) and (l) compressive modulus (m) of muscle and fat fibers in the commercial meat (white bar) and TIP-derived (gray bars). The modulus of the muscle fiber on day 3 of differentiation (after 6 days) and the fat fiber on day 7 of differentiation (after 10 days) was measured (n = 3 independent samples, paired one-way ANOVA with a Tukey’s HSD post test (l) and pairwise t-test comparison (m)). *p<0.05, **p<0.01, ***p<0.001; error bars represent mean ± s.d. Representative images from at least two independent experiments are shown. Source data are provided as a Source Data file.
was found six times smaller than the meat fibers (Fig. 4I), indicating that optimization of the bSC concentration or the ECM concentration in the bioink will be necessary to be equivalent to the real meat.

Although the water content showed the disparity between the commercial beef and the TIP-derived cell fibers (Supplementary Fig. 21), while compressive modulus in all cell fibers (muscle fiber on day 3 of differentiation and fat fiber on day 7 of differentiation) showed similar values, which were within one order of kPa (Fig. 4m and Supplementary Fig. 22). Since the TIP-derived cell fibers were not controlled for tenderness, flavor, and additional nutrient components in this study, these factors will need to be addressed to produce customer-oriented cultured meat.

**Engineered steak construction by assembly of muscle, fat, and vascular cell fibers.** The assembly of the TIP-derived cell fibers was attempted to demonstrate the construction of the cultured steak. To mimic the structure of commercial beef, we first took a cross-sectional image of Wagyu with sarcomeric α-actinin (blue) and laminin- (brown) stained image (left) of the commercial meat. It is assumed that the diameters of the fibrous muscle, fat, and vascular tissues are about 500, 760, and 600 µm, respectively. Scale bar, 1 mm. 

![Image of Wagyu](https://example.com/wagyu_image) (Fig. 4m and Supplementary Fig. 22). Since the TIP-derived cell fibers were not controlled for tenderness, flavor, and additional nutrient components in this study, these factors will need to be addressed to produce customer-oriented cultured meat.

The use of ADSCs for their endothelial differentiation allowed us to avoid the direct isolation of bovine endothelial cells, which could have added a limiting step to the full process. Moreover, their differentiation in adipocytes, while previously done, still remains little studied. Especially, the induction of the late marker FABP4 was not reported, while its role in the bovine adipogenesis was indeed found of importance for the bovine lipid metabolism-related gene induction. Our study thus provided additional information on both adipogenic and endothelial differentiations from bovine stem cells, which can have other further applications in the meat-related fields.

Furthermore, it was then shown that the resistance to the contraction force during the culture of the bSC-derived cell fiber was essential to realize highly aligned muscle fibrils (Fig. 3c–e). A
modified supporting bath-assisted cell printing method, the tendon-gen-integrated printing (TIP), was thus developed, in which the collagen gel-based tendon tissues can withstand the cell traction force during the bSCs differentiation, leading to a well-maintained fibrous structure and its cell alignment (Fig. 4a–f). Comparisons of the cell density (Fig. 4i), compressive modulus (Fig. 4m), and water content (Supplementary Fig. 21) showed the gap between TIP-derived and commercial muscle and fat cell fibers. We demonstrated engineered steak-like tissue, analogous to the structure of commercial beef, through the manual assembly of muscle, adipose, and blood capillary cell fibers produced through the TIP (Fig. 5a–c). Up to our knowledge, this is the first report to demonstrate the fabrication of whole-cut cultured meat-like tissue that was composed of three types of primary bovine cells isolated from an edible meat block and was modeled into a real meat’s structure. Since the demonstrated steak-like tissue is a small piece and inedible, further elaboration will therefore be required with consideration of TIP-based cell printing scalability and edibility of culture and cell-printing-related materials in the future. In addition to primary bovine cells for cultured meat, we expect that the TIP will also benefit the muscle tissue engineering applications in the future.

Methods

Isolation and purification of bSCs and bADSCs. bSCs were isolated from 160 g of fresh masster muscle samples (within 8 h of euthanasia) of 27-month-old Japanese black cattle obtained at the slaughterhouse (Tokyo Shibaura Zouki, Tokyo, Japan, and JA ZEN-NOH Kanagawa, Kanagawa, Japan). The freshly harvested bovine muscle was kept on ice, transferred to a clean bench, and washed with cold 70% ethanol for 1 min, followed by cold PBS 1 x 2 times. Then, the fat tissue part was disposed of, the remaining muscle was cut into small pieces with a knife, and minced with a food processor mechanically. The bovine minced muscle was washed with cold PBS 1 x 1% penicillin-streptomycin (PS; Lonza, 17-4745) for 1 min. The washed muscle was transferred to a bottle and mixed with 160 ml of 0.2% collagenase II (Worthington, CL-2) in DMEM (Invitrogen, 41966-29) supplemented with 1% PS. The bottle was incubated and shaken every 10 min for 1.5 h at 37 °C. After digestion, 160 ml of 20% FBS in DMEM supplemented with 1% PS was added to the solution, and the mixture was centrifuged for 3 min at 80 g and 4 °C. Floating tissues in the supernatant after centrifugation were removed by tweezers and then the collected supernatant was kept on ice as a mononuclear cell suspension. Precipitated debris were mixed with 80 ml of cold 1% PS in PBS 1 x and centrifuged for 3 min at 80 g and 4 °C. The supernatant was collected again and mixed with the remaining cell suspension. After that, the suspension was filtered through a 100 µm cell strainer. After centrifugation for 5 min at 1500 g and 4 °C, the cells were suspended with 160 ml of cold DMEM with 20% FBS and 1% PS, were filtered through a 100-µm cell strainer followed by a 40-µm cell strainer. The cells were then centrifuged for 5 min at 1500 g and 4 °C. Precipitated cells were incubated with 8 ml of erythrocyte lysis buffer (ACK; 786-850) for 5 min on ice, then the cells were washed twice with cold PBS 1 x supplemented with 1% PS and the cell pellet was mixed with PBS supplement with 10% dimethyl sulfoxide and then reserved at ∼150 °C. The frozen cells were recovered in a 37 °C water bath and washed with cold PBS 1 x twice. The cells were suspended in F10 medium (Gibco, 31530-010) supplemented with 10% FBS, 100 ng/ml bFGF (R&D, 233-FB-025), and 1% PS, and then filtered through a 40-µm filter holder, microsyringe 25-mm filter holder, Merck, before being freeze-dried for 18 h (FDU-2200, YELELA). The obtained cell was kept in a desiccator at RT.

Collagen microfiber preparation. The collagen microfibers (CMF) were first weight and washed in DMEM without FBS by being centrifuged for 1 min at 1970 g to get a final cell concentration of 5 x 10^6 cells/ml. The pellet containing CMF and bADSCs was then mixed in a fibrinogen (Sigma Aldrich, F8630) solution at 6 mg/ml final concentration (the stock solution was at 50 mg/ml), 10% DMEM with 1% PS, and the thrombin solution (Sigma Aldrich, T4648) was added to get a final concentration of 3 U/ml (the stock solution at 200 U/ml) prepared in DMEM with 10% FBS and 1% antibiotics). Finally, 2 µl drop tissues were seeded in a 96 well plate (Iwaki, 3860-096) and gelated for 15 min in the incubator at 37 °C. Then 300 µl of medium (DMEM with 10% FBS and 1% antibiotics) was added to the drop tissues. For adipogenic differentiation, three days of proliferation were first necessary to allow the bADSCs proliferation, until reaching a suitable cell–cell interaction required for the adipogenesis. The medium was then switched for DMEM with 10% FBS containing different adipogenic components to compare: Rosiglitazone (at 20 µM final concentration, Sigma Aldrich, R2408), Insulin (at 10 µg/ml final concentration, Sigma Aldrich, I6634), Tretinogline (at 40 µM final concentration, Sigma Aldrich, T2573), Pristane acid (at 50 µM final concentration, Funakoshi, 11-1500), Phytic acid (at 50 µM final concentration, Sigma Aldrich, P4060), Erucic acid (at 50 µM final concentration, Sigma Aldrich, 45629-F), Elaidic acid (at 50 µM final concentration, Sigma Aldrich, 45089), Oleic acid (at 50 µM final concentration, Sigma Aldrich, O1383), Palmitoleic acid (at 50 µM final concentration, Sigma Aldrich, 76169), Myristoleic acid (at 50 µM final concentration, Sigma Aldrich, 41788), TG family type receptor activin-like kinase 5 inhibitor (ALK5 II, 2-[3-(6-methyl-2-pyridinyl)-1H-pyrazol-4-yl]-1H-pyrindine-1-yl, at 1–10 µM final concentration, Cayman, 14794), Endothelial Cell Growth Factor (Eli Lilly and Company, Endothelial Cell Growth Factor, Ewen II Fly II), and Collagenase (Merck KGaA, EMD Millipore, 01501). The obtained cell line seven free fatty acids were compared as well as some other possible different mixtures (pristanic and phytanic acids together, or the other five remaining free fatty acids) following already published possible adipogenesis inducers for bovine ADSCs. The 300 µl of differentiation medium was then renewed every 2-3 days.

3D gel-embedded culture. To construct the adipose tissues by 3D culture, CMF were first weight and washed in DMEM without FBS by being centrifuged for 1 min at 16,083 g to get a final concentration in the tissues of 1.2 wt%. The bADSCs were added after trypsin detachment (always used at P1–5) and centrifuged for 1 min at 1970 g to get a final cell concentration of 5 x 10^6 cells/ml. The pellet containing CMF and bADSCs was then mixed in a fibrinogen (Sigma Aldrich, F8630) solution at 6 mg/ml final concentration (the stock solution was at 50 mg/ml), 10% DMEM with 1% PS, and the thrombin solution (Sigma Aldrich, T4648) was added to get a final concentration of 3 U/ml (the stock solution at 200 U/ml) prepared in DMEM with 10% FBS and 1% antibiotics). Finally, 2 µl drop tissues were seeded in a 96 well plate (Iwaki, 3860-096) and gelated for 15 min in the incubator at 37 °C. Then 300 µl of medium (DMEM with 10% FBS and 1% antibiotics) was added to the drop tissues. For adipogenic differentiation, three days of proliferation were first necessary to allow the bADSCs proliferation, until reaching a suitable cell–cell interaction required for the adipogenesis. The medium was then switched for DMEM with 10% FBS containing different adipogenic components to compare: Rosiglitazone (at 20 µM final concentration, Sigma Aldrich, R2408), Insulin (at 10 µg/ml final concentration, Sigma Aldrich, I6634), Tretinogline (at 40 µM final concentration, Sigma Aldrich, T2573), Pristane acid (at 50 µM final concentration, Funakoshi, 11-1500), Phytic acid (at 50 µM final concentration, Sigma Aldrich, P4060), Erucic acid (at 50 µM final concentration, Sigma Aldrich, 45629-F), Elaidic acid (at 50 µM final concentration, Sigma Aldrich, 45089), Oleic acid (at 50 µM final concentration, Sigma Aldrich, O1383), Palmitoleic acid (at 50 µM final concentration, Sigma Aldrich, 76169), Myristoleic acid (at 50 µM final concentration, Sigma Aldrich, 41788), TG family type receptor activin-like kinase 5 inhibitor (ALK5 II, 2-[3-(6-methyl-2-pyridinyl)-1H-pyrazol-4-yl]-1H-pyrindine-1-yl, at 1–10 µM final concentration, Cayman, 14794), and Collagenase (Eli Lilly and Company, Endothelial Cell Growth Factor, Ewen II Fly II). The obtained cell line seven free fatty acids were compared as well as some other possible different mixtures (pristanic and phytanic acids together, or the other five remaining free fatty acids) following already published possible adipogenesis inducers for bovine ADSCs. The 300 µl of differentiation medium was then renewed every 2-3 days.
removing the supernatant. G-Gel was produced by preparing 4.5 wt% porcine gelatin (Sigma Aldrich, G1890) in DMEM containing 1% antibiotic-antimycotic mixed solution (100X, Invitrogen, 15220-018) in PBS, putting it at 4 °C overnight for 24 h, and the reaction products were visualized in blue (Vector Laboratories, Vector Blue) and brown (DAKO, DAB+, chromogen), respectively.

**Immunostaining.** Immunostaining was conducted by a general process, 4% paraformaldehyde fixation at RT for 15 min or at 4 °C overnight, permeabilization was performed by 0.5% Tween 20 + 0.2% Triton X-100 (Sigma Aldrich, T8787) at RT for 15 min, blocking with 1% BSA (Sigma Aldrich, A2870) for 30–60 min, incubation with the 1st antibody in 1% BSA at RT for 2 h or at 4 °C overnight, incubation with cocktails containing fluorophore-conjugated 2nd antibody and 1 µg/mL TRITC-phalloidin (Sigma Aldrich, P-1551) for actin staining or 100 ng/mL NileRed (TCl, No659) for lipid staining at RT for 1 h, and finally 300 nM DAPI (Invitrogen, D41490) for nucleus staining. Myosin 4 Monoclonal Antibody (eBioscience, 14-6503-82, MF20, dilution 1/500) for bSCs, Anti-Cd31 (Wako, M0823, JCT0A, dilution 1/100) for bovine endothelial cells, and PPAR gamma (Abcam, ab45036, polyclonal, dilution 1/100) and FABP4 (LSBio, LS-B4247, polyclonal, dilution 1/100) antibodies for bovine adipocytes were used as 1st antibodies. Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (Invitrogen, A-11001, polyclonal, dilution 1/200) and goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 (Invitrogen, A-21235, polyclonal, dilution 1/200) were used as 2nd antibodies. All fluorescence imaging were taken by confocal microscopy. In the case of printed cell fibers, they were treated with Rapiclear 1.52 (SUNJin Lab) at RT for 30 min for deep-tissue imaging.

**Rheology measurement.** Viscosity was measured by the controlled-rate mode of the rheometer (Thermo Scientific, HAAKE Rheostress 6000) to verify the thixotropy of G-Gel and G-GG. The steps were composed at 0.01%/s for 30 s, at 0.1%/s for 100 s, and at 0.01%/s for 30 s.

**UV-Vis.** The disposable cuvette was filled with 1 mL of 4 wt% CNFs, and then transmittance was measured at the temperature-controlled steps by UV–Vis spectrometer (Jasco, V-670) at 4 °C for 2000 s, at 37 °C for 2000 s, at 4 °C for 2000 s, and then at 37 °C for 2000 s. After each temperature change, the photos of the samples were taken.

**Mechanical test.** The elastic modulus of these printed fibers was measured with EZ test (SHIMADZU, EZCE 500 N) to verify the thixotropy of G-Gel and G-GG. The steps were composed at 0.01%/s for 30 s, at 0.1%/s for 100 s, and at 0.01%/s for 30 s.

**DNA content measurement.** Commercial beef was bought from the supermarket and intramuscular fat tissues as well as muscle tissue parts were cut into small fibers of the same size as the printed fibers (Fig. S19). One fiber from the commercial fibers or printed fibers was put per microcentrifuge tube and the tissues were lysed following the DNeasy Blood & Tissue Kit (QIAGEN, 69504) to extract their DNA content, which was quantified by the NanodropTM N1000 device (Thermo Fisher Scientific). Then the DNA amount was presented normalized by the weight of the samples before lysis.
MHC-positive area was measured by ImageJ. For the measurement of cell alignment degree, we randomly selected the fluorescence images (actin stained with TRITC–Phalloidin T-Stack imaging of one 3D-printed tissue, overlaid the same images showing clear-cell morphologies, and measured the angle of individual cells to printed cell fiber’s major axis by using ImageJ. The number of measured cells in each condition was in the range of 27–36. For the calculation of lipid production from bADSCs, the 3D tissues’ Z-stack images were taken (3 slices with 50 μm step) with the same exposure time, brightness, and contrast, then the summation of Z-slice’s intensity in Nile Red and Hoechst of each 3D tissue was done by Image J. The relative intensity was calculated from the total Nile Red’s intensity divided by the Hoechst intensity in each 3D tissue. The same experiments were repeated for each condition compared. The 3D-reconstructed image and the printed cell fibers’ 3D movie was obtained by Imaris software (Bitplane).

Gene expression. Gene expression was analyzed using real-time quantitative polymerase chain reaction (RT-qPCR). Adipose drop tissues at days 0, 7, and 14 of differentiation, as well as bioprinted fiber samples at days (before bioprinting) and at days 6 (myoblast fibers), 7 (endothelial fibers), or 14 (adipocyte fibers) were washed out in PBS and total RNA extraction was carried out using the PAXgene RNA Micro Kit (InVitrogen, Waltham, USA), with the DNAse step, following the manufacturer’s instructions. Samples’ RNA content was quantified with the NanodropTM spectrometer (N1000, Thermo Fisher Scientific, Waltham, USA). For RT-qPCR, the RNA samples were first submitted to reverse transcription into cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, USA), before being quantified by a BCA Protein Assay Kit (23225, ThermoFisher Scientific). About 4–10 μg of protein was resolved on each lane of 4–15% protein gels (Mini–PROTEAN® TGE Stain-Free® 4568084, BIORAD with Running Buffer for SDS-Tris-Glycine (10X), Cosmobil, electrotransferred onto a PVDF membrane (Immun–Blot® 1620-0176 BIORAD), and probed using specific antibodies: Myosin, a 4 Monoclonal antibody (Ebioscience, 14-5600-82, dilution 1/1000), PPAR gamma antibody (Abcam, ab54306, monoclonal, 1/1000), FABP4 antibody (LSBio, LS-B4227, polyclonal, dilution 1/1000), and β-Actin antibody (Sigma-Aldrich, A5441, AC-15, dilution 1/3000). Proteins were detected by secondary antibodies conjugated to horseradish peroxidase (Anti–Mouse IgG 170-4516, anti-Rabbit IgG 179-6515, dilution 1/5000, and Precision Protein™ StreptTactin-HRP Conjugate 161-0380, BIORAD, dilution 1/5000). Signal detection was performed by an enhanced chemiluminescence-detection reagent (Clarity Western ECL Substrate 1705060, BIORAD) using the ChemiDoc Imaging System (BIORAD). Molecular weights were determined by comparison with the migration of a protein standards (Precision Plus Protein Standards-130 KDa KaleidoscopeTM Standards 161-0395, BIORAD). Quantitative estimation of the bands’ intensity was performed using ImageJ software.

Statistical analysis. Statistical analysis was performed using EZAnova (version 0.98, University of South Carolina, Columbia, SC, USA) and GraphPad Prism 8 (Version 8.4.3 (686), San Diego, USA) software. The detail of the number of n corresponding to the number of independent experiments using isolated bovine primary cells from different bovine donors, or independent samples, is displayed on each graph in the figures and in the captions, as well as the exact p values. For paired samples when the same cells were measured at different times (Fig. 2f and h), a one-way ANOVA with a repeated measures design was used, with the Greenehouse–Geisser correction and the Tukey’s HSD post test for the multiple comparisons. When two different treatments were applied to the cells (Fig. 2i), a classic two-ways ANOVA was performed, with the Sidak post test for the multiple comparisons. ANOVA was used when more than two conditions were compared with each other and for only two conditions, the pairwise t-test comparison was performed (Fig. 4f, i, j, m, Supplementary Fig. 17, and Supplementary Fig. 21). EZAnova software was used only for Fig. 2d analysis, for the other statistical analysis, GraphPad Prism software was used. Error bars represent SD. p values were considered significantly different at least when p < 0.05. When no marks are shown on the graphs, it means that the differences are not significant.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
All relevant data supporting the key findings of this study are available within the article and its Supplementary Information files or from the corresponding author upon reasonable request. Source data are provided with this paper.

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Author contributions
D.-H.K. and M.M. contributed to design the study and mainly draft the paper; D.-H.K., F.L., and H.L. carried out the cell culture, bioprinting experiments, and biological analysis; F.L. and H.L. also contributed to draft the paper; H.S. conducted the histological experiment and analysis; Y.N., H.N., and M.K. provided practical advice and primary cultured bovine cells; D.T., D.K., S.I., and S.K. provided technical guidance and advice on bioprinting instruments; E.N. provided critical advice on muscle biology;

Competing interests
The authors D.-H.K., F.L., S.I., S.K., and M.M. declare the following financial competing interests: D.-H.K. and M.M. have filed the patent (PCT/JP2021/014194) related to the cell-printing technique described in the paper. F.L., S.I., S.K., and M.M. have filed the patents (PCT/JP2018/041659 and PCT/JP2020/012476) related to the adipogenesis of adipose-derived stem cells. F.L., S.K., and M.M. have filed the patent application (JP2020-196209) related to the endothelial differentiation of adipose-derived stem cells described in this paper. This research was also supported by TOPPAN INC. The remaining authors declare no competing interests.

Additional information
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