2.4. Cell sheet retrieval

A polyisopropylacrylamide (PIPAAm) membrane was fixed to the surface of the temperature-responsive culture dishes. This enabled the dish surface to reversibly change between a cell-adhesive hydrophobic surface and a cell-releasing hydrophilic surface at a temperature threshold of 32 °C. Hence, by lowering the temperature, the complete cell sheet could be harvested from the dish while maintaining the sheet structure, without requiring the use of enzymes such as trypsin, which would damage the cells.

Cell sheets harvested in this manner maintain the extracellular matrix, thus enabling a nonsuture-based transplantation procedure. Furthermore, to create a layered cell sheet, the cell sheet was covered with a polyvinylidene difluoride (PVDF) membrane, and then the cell sheet and the PVDF membrane were harvested carefully as one unit. More exactly, the PVDF membrane that covered the cell sheet was harvested by rolling the membrane up at the edge of the culture dish and then overlaying the membrane on top of another cell sheet. By repeating this procedure three times, a triple-layered chondrocyte sheet was obtained.

2.5. Transplantation of synovial cells and layered chondrocyte sheets

We used 16-week-old wild-type male Lewis rats as allograft recipients. The transplantation procedure was carried out under general gas anesthesia using 2% isoflurane in O_2. On the right side, a medial parapatellar incision was made and the patella was moved aside slightly, opening the knee cavity to expose the patellar groove of the femur. We then created osteochondral defects (φ, 3 mm) with an 18-gauge needle (it is known that natural restoration is impossible in defects of this size). We then transplanted the harvested luciferase-expressing chondrocyte sheet to the defect.

The characteristics of individual chondrocyte and synovial cell sheets and the synergistic effect achieved by combining these sheets were determined from the cell counts obtained from two-layered luciferase-expressing chondrocyte sheets (AC-AC group), two-layered luciferase-expressing synovial cell sheets (SY-SY group), and sheets comprised of both types of luciferase-expressing sheets (AC-SY group). These sheets were constructed and then transplanted to the osteochondral defects in the right knees of wild-type Lewis rats ($n = 12$, for each type of sheet).

2.6. In vivo BLI

After the bioengineered sheets were transplanted, the rats were repeatedly imaged using BLI with an IVIS system (Xenogen Corp; Hopkinton, MA, USA) to detect photons emitted by the transplanted luciferase-expressing sheet cells. For BLI, the rats were anesthetized with isoflurane and subcutaneously injected with luciferin (150 mg/kg of body weight; D-luciferin, Biosynth AG, Staad, Switzerland) near the scapula. The method of administering luciferin is described in detail below. These rats were then placed in a light-tight chamber for imaging using a CCD camera. The photon emission from the peak luciferase activity was recorded and the BLI images were aligned using the IGOR and IVIS Living Image software packages.

2.7. Comparison of several methods of luciferin injection

To determine the best method of administering luciferin, we compared three alternatives: intravenous injection (IV), subcutaneous injection (SC), and intra-articular injection (IA). We administered luciferin to the same Lewis rats to which luciferase-expressing chondrocyte cell sheets were transplanted. In all three methods, we administered luciferin at a concentration of 50 mg/ml, which is close to the saturation point. In the IV method, we administered luciferin solution at 60 mg/kg through the penile vein. In the SC method, we administered luciferin solution at 150 mg/kg near the scapula, and for the IA method, we injected luciferin at 30 mg/kg directly into the knee joint cavity under the patella. All treatments were carried out under isoflurane anesthesia. After injection, the rat was quickly placed in a light-tight chamber equipped with a CCD camera, and light emission was measured at intervals of 1 min. The change in luminescence intensity over time was monitored. Each injection method yielded differing dynamic profiles of photon emission (Fig. 1A–C).

When using the IV injection method, the amount of time required for the luminescence intensity to reach peak levels was extremely short (1–2 min after injection) and the intensity declined rapidly thereafter (Fig. 1A). Consequently, in some cases, photon emission could only be measured after the peak level had been reached. In addition, injection into the penile vein was technically challenging; if not done correctly, the vein would swell, making it difficult to repeat the injection procedure.

Following SC injection, the luminescence intensity rose gradually and peaked 20–30 min after injection, after which it declined gradually (Fig. 1B). This technique was not technically challenging; however, the method required a large amount of luciferin. Achieving the same intensity with the SC method required more than twice the dosage of luciferin used in the IV method.

Following IA injection, the luminescence intensity rose to peak levels after 4–5 min (Fig. 1C). Compared with the IV method, the IA injection was technically easier to carry out. However, in some cases, the technique failed. The technique allowed luciferin to be directly delivered to luciferase-expressing cells without having to enter the bloodstream. Of the three techniques, the IA method allowed for the maximum emission strength to be achieved using the smallest amount of luciferin. Even though this method required only 0.2 ml of drug solution, it nevertheless resulted in swelling of the articular capsule of the knee joint and increasing synovial pressure, leading to a concern about the effect that the swelling might have on the transplanted cells or the regeneration of the osteochondral defect. Of the three methods, we found that SC injection allowed us to measure the peak levels of intensity by viable luciferase-expressing cells, while at the same time being the least technically challenging method, and with minimal undesirable side effects on the knee joint.

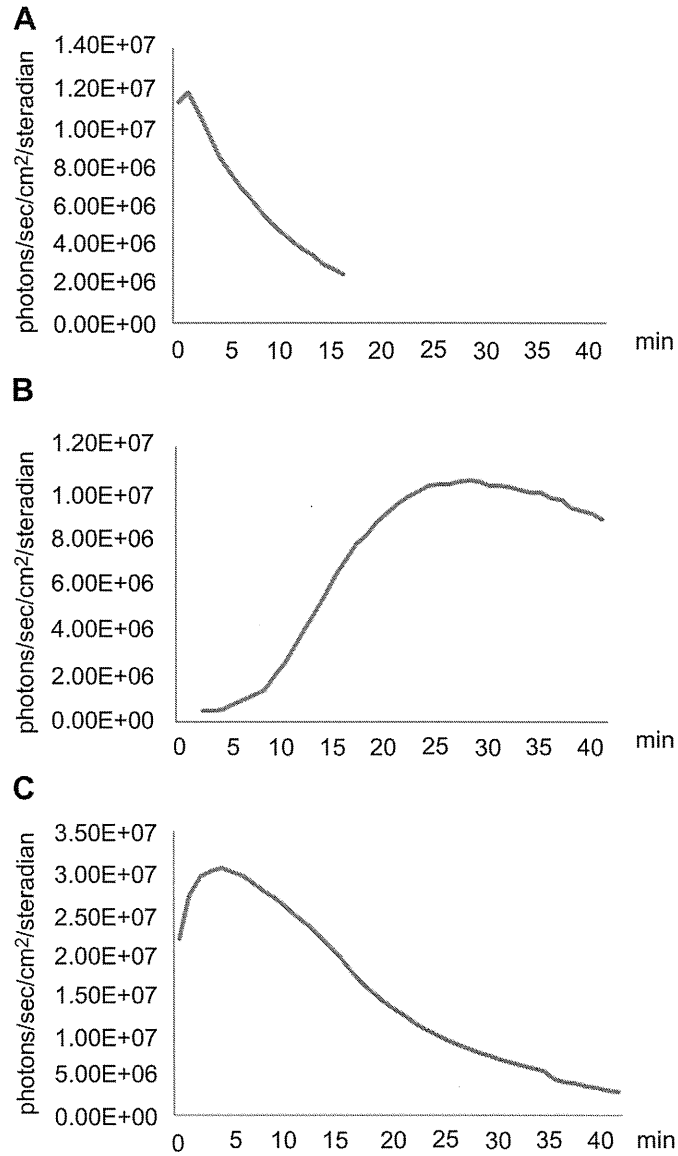

Fig. 1. Time course of BLI signals after IV (A), SC (B), or IA (C) injection of luciferin in rats. IV, intravenous injection; SC, subcutaneous injection; and IA, intra-articular injection.

2.8. Histological evaluation and scoring of the repair of osteochondral defects

We previously demonstrated in experiments with Japanese white rabbits that transplantation of cell sheets made of a combination of chondrocytes and synovial cells had a superior regenerative effect on osteochondral defects compared with those achieved with chondrocyte sheets or synovial cells alone [16]. We carried out similar experiments on Lewis rats in this study.

We used 24 of the aforementioned 36 wild-type Lewis rats that received cell sheet allografts for the following experiment ($n = 8$, per group). Two rats from each of the groups were euthanized by inhalation of CO_2 at 2, 4, 6, and 8 weeks after transplantation, and a specimen obtained from the site of the osteochondral defect on the transplanted knee was processed as follows. The tissue samples were fixed in 4% paraformaldehyde for 1 week. After decalcification for 2–3 weeks using distilled

Table 1
ICRS histological grading system.[a]

Ti
Tissue morphology
Lateral integration of implanted material

4: mostly hyaline cartilage
3: mostly fibrocartilage
2: mostly noncartilage
1: exclusively noncartilage

Matx
Matrix staining

1: none
2: slight
3: moderate
4: strong

Stru
Structural integrity

1: severe disintegration
2: cysts or disruptions
3: no organization of chondrocytes
4: beginning of columnar organization of chondrocytes
5: normal, similar to healthy mature cartilage

Clus
Chondrocyte clustering in implant

1: 25–100% of the cells clustered
2: <25% of the cells clustered
3: no clusters

Tide
Intactness of the calcified cartilage layer, formation of tidemark

1: <25% of the calcified cartilage layer intact
2: 25–49% of the calcified cartilage layer intact
3: 50–75% of the calcified cartilage layer intact
4: 76–90% of the calcified cartilage layer intact
5: complete intactness of the calcified cartilage layer

Bform
Subchondral bone formation

1: no formation
2: slight
3: strong

SurfH
Histological appraisal of surface architecture

1: severe fibrillation or disruption
2: moderate fibrillation or irregularity
3: slight fibrillation or irregularity
4: normal

FilH
Histologic appraisal of defect-filling

1:<25%
2: 26–50%
3: 51–75%
4: 76–90%
5: 91–110%

Latl
Lateral integration of implanted material

1: not bonded
2: bonded at one end/partially at both ends
3: bonded at both sides

Basl
Basal integration of implanted material

1: <50%
2: 50–70%
3: 71–90%
4: 91–100%

InfH
Inflammation

1: no inflammation
3: slight inflammation
5: strong inflammation

Hgtot
Histological grading system
Some of the histological variables:

- tissue morphology(Ti)
- matrix staining (Matx)
- structural integrity (Stru)
- cluster formation (Clus)
- tidemark opening (Tide)
- bone formation (Bform)
- histological surface architecture(SurfH)
- histological degree of defect filling (FilH)
- lateral integration of defect-filling tissue (Latl)
- basal integration of defect-filling tissue (Basl)
- histological signs of inflammation (InfH)

Maximum total: 45 points

[a] The ICRS system evaluates repair tissue based on 11 histological variables: tissue morphology (Ti), matrix staining (Matx), structural integrity (Stru), cluster formation (Clus), tidemark opening (Tide), bone formation (Bform), histological appraisal of surface architecture (SurfH), histological appraisal of the degree of defect filling (FilH), lateral integration of defect-filling tissue (Latl), basal integration of defect-filling tissue (Basl), and histological signs of inflammation (InfH). The grades for each variable were then combined to yield an overall histologic grading value (Hgtot). The total scores range from 11 to 45.

water (pH 7.4) containing 10% ethylenediaminetetraacetic acid (EDTA), the tissue was embedded in paraffin wax and sectioned perpendicularly through the center of the defect. Each section was stained with Safranin O for glycosaminoglycans for histological evaluation.

The International Cartilage Research Society (ICRS) histological grading system for evaluating tissue repair was used to assess the defect sites. This newly revised system [26–28] (Table 1), developed by O'Driscoll, Keeley and Salter, includes 11 histological variables: morphology (Ti), matrix staining (Matx), structural integrity (Stru), cluster formation (Clus), tidemark opening (Tide), bone formation (Bform), histological appraisal of surface architecture (SurfH), histological appraisal of the degree of defect filling (FilH), lateral integration of defect-filling tissue (Latl), basal integration of defect-filling tissue (Basl), and histological signs of inflammation (InfH). The assigned scores for each of these 11 histological variables range from 11 to 45 points. In our experiments, the variables were scored by two individuals.

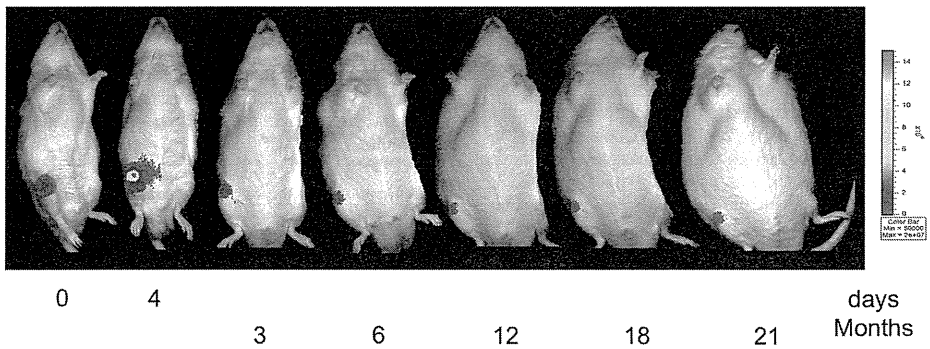

Fig. 2. CCD images from the long-term observation of luciferase activity from the right knee joint. This representative image shows the CCD image of one rat from the AC-SY group (transplanted with chondrocyte and synovial cell sheets) at the following time points (from left to right): after transplantation on day 0 and day 4, and at 3, 6, 12, 18, and 21 months. The color bar indicates the bioluminescence intensity in photons per seconds cm^2 per steradian.

2.9. Statistical analyses

The Kruskal–Wallis and Mann–Whitney *U* tests were used for the statistical analyses. *P*-values <0.05 were considered significant.

3. Results

3.1. Bioluminescence imaging

Photon emission from the right knee joints of the rats in the long-term observation group was visible by BLI for more than 21 months after graft transplantation (Fig. 2). The BLI signal was not detected anywhere other than the right knee, to which the bio-engineered chondrocyte sheets had been transplanted.

We confirmed the above observations in all but one rat in the synovial group that died 8 days after transplantation. Moreover, we also did not observe any apparent motor dysfunctions in rats in any of the groups.

3.2. Changes in luminescence intensity over time

The intensity of light emission of each group over a period of 21 months, with day 0 set as 100%, is shown in Fig. 3. The luminescence intensity of all groups peaked 3–4 days after transplantation, at which point the intensity of the AC-AC, AC-SY, and SY-SY groups was respectively 16, 5, and 7 times that on the transplantation date. However, in all groups, the intensity decreased gradually over 3–4 weeks, after which it stabilized at approximately 1/10 of the intensity on the transplantation date (Fig. 3). In other words, the intensity decreased in the three groups to values that were 1/160, 1/50, and 1/70 of the peak intensity,

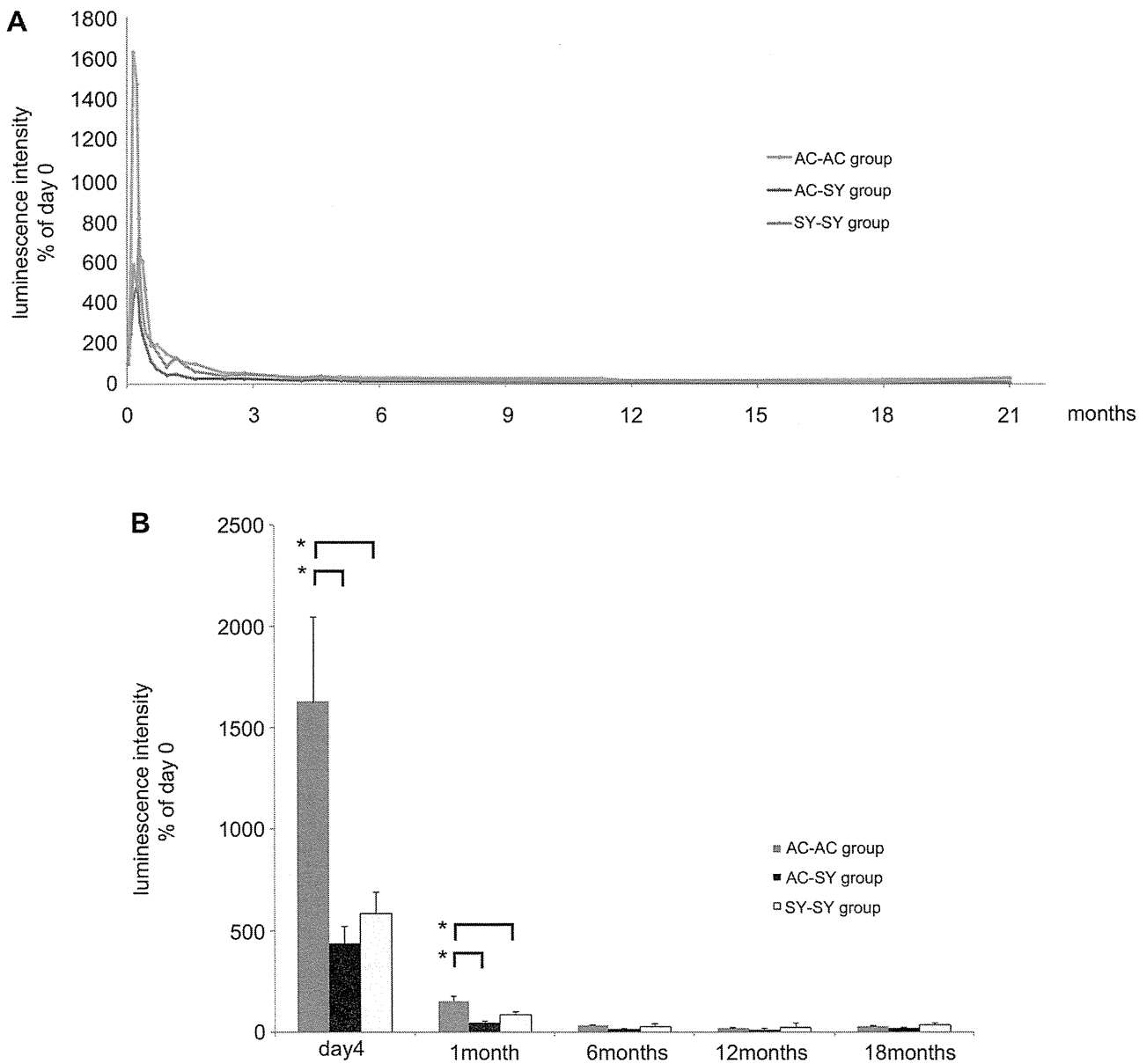

Fig. 3. Sequential quantification of luminescence intensity. Changes in the intensity of the light emission of the transplanted cells were monitored over 21 months, with the intensity on the day of transplantation being set to 100% (A). Bar graph showing the percent change in luminescence intensity in the three groups at several points after transplantation (B). Significant differences were observed between the AC-AC and AC-SY groups and the AC-AC and SY-SY groups, at 4 days and 1 month after transplantation, respectively ($p < 0.05$, *). Error bars represent standard errors. AC-AC group, transplanted with two chondrocyte sheets; SY-SY group, transplanted with two synovial cell sheets; and AC-SY group, transplanted with chondrocyte and synovial cell sheets.

respectively. However, the luminescence continued to persist at this lower value without disappearing for more than 21 months (Fig. 3A). The differences in luminescence intensity between the three groups were significant at 4 days and 1 month after transplantation. However, significant differences were not observed in the long term (Fig. 3B).

3.3. Histological evaluation and scoring for the repair of osteochondral defects

Changes in the histological features of the region encompassing the osteochondral defects were evaluated 2, 4, 6, and 8 weeks after transplantation (Fig. 4A). The ICRS grading system scores for

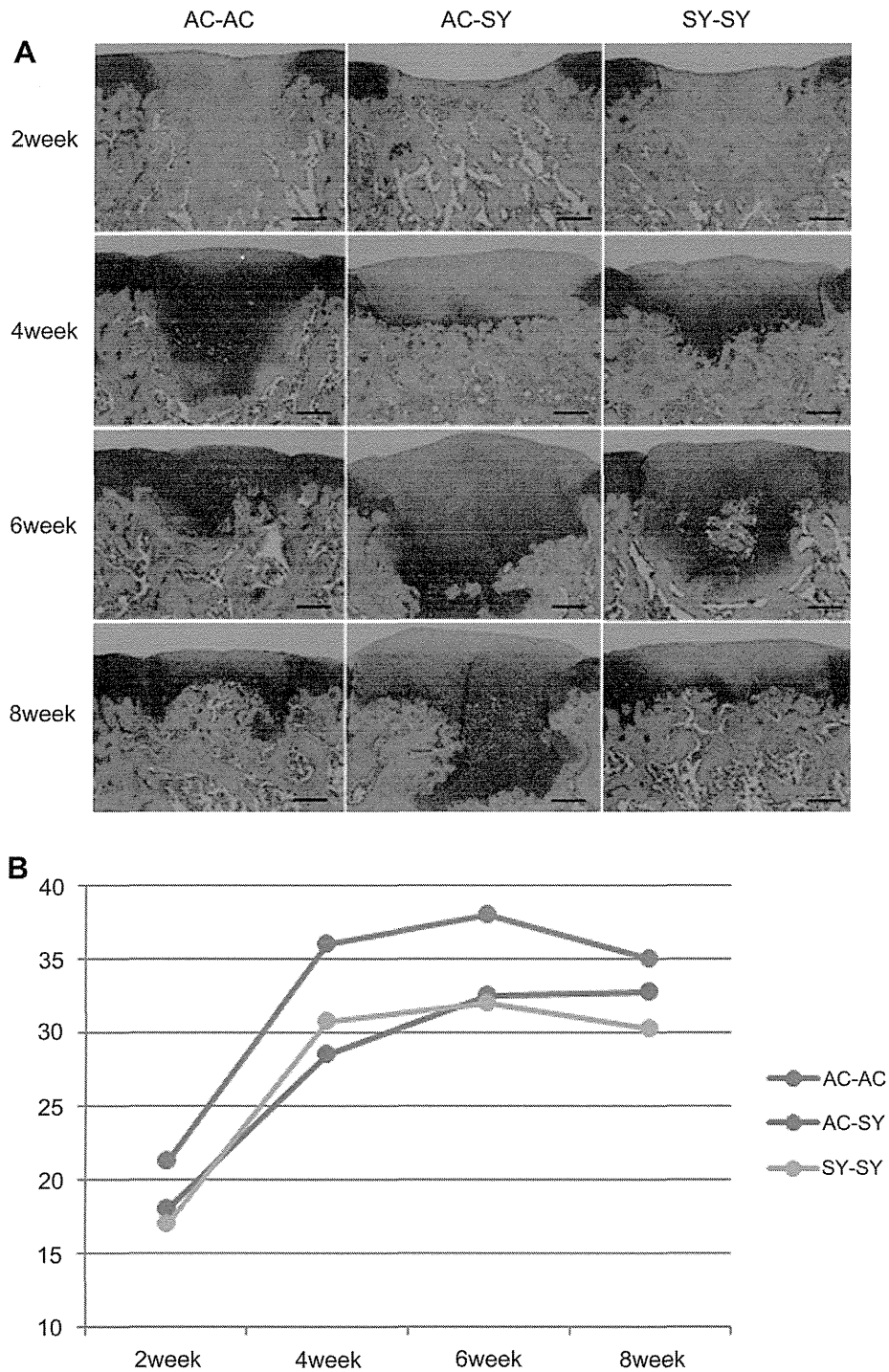

Fig. 4. Histological changes in the repair tissue 4–8 weeks after transplantation. Tissue findings (A) are stained with Safranin O. The scale bar represents 500 μm. Changes in the ICRS grading score after transplantation over time are plotted on a line chart (B). The data shown are the average ICRS grading scores for tissue repair in two rats. AC-AC group, transplanted with two chondrocyte sheets; SY-SY group, transplanted with two synovial cell sheets; and AC-SY group, transplanted with chondrocyte and synovial cell sheets ($n = 2$, per group).

regeneration of osteochondral defects at 2, 4, 6, and 8 weeks after transplantation are shown in Fig. 4B. During this period of observation, the score of each group was the highest at 6 weeks. At 8 weeks, we observed a decrease in the scores of the AC-AC and SY-SY groups. The scores for the AC-SY group remained unchanged from week 6 onward.

4. Discussion

In this study, we successfully monitored the long-term survival of bioengineered chondrocyte and synovial cell sheets by cell tracking using BLI. This method is a noninvasive, longitudinal approach for *in vivo* monitoring of transplanted cells without the need to euthanize recipient animals. Our data show that the transplanted cell sheets can survive in the knee joint for more than 21 months. Furthermore, we confirmed that the cells in the allografted sheets remained at the transplantation site and did not migrate from the knee joint to any other location.

The chondrocyte sheet is an essential initiator, providing the signals and humoral factors for cartilage formation, and tissue repair and regeneration. The initial, effective repair by host cells can be achieved after transplantation of this sheet [7–9]. We thought that transplanted chondrocyte sheets would disappear from the site of the cartilaginous defects in the knee joints within 3–4 weeks. However, our results indicate that it is possible that the defect site may function as an environmental niche appropriate for the bioengineered chondrocyte sheets, and that the surviving grafted cells acquire a dedifferentiated phenotype. The grafted stem cells may thus contribute to cartilage tissue regeneration. To confirm and extend this observation, further studies are required in other models of cartilage repair and regeneration.

We evaluated the survival duration of cell sheets composed of a combination of chondrocytes and synovial cells. In previous experiments, we demonstrated that, compared with cell sheets composed of only chondrocytes, the combination sheets were more effective in promoting the regeneration of subchondral bone and the formation of chondrocyte postregeneration [16]. Therefore, we expected that the combination of the two types of cells would result in a longer survival duration than the use of either cell type alone. However, in rats transplanted with any of the three types of cell sheets, the transplanted cells survived for more than 21 months. Therefore, it is not clear what effect, if any, using a cell sheet made of a combination of the two cell types has on survival duration.

We observed a peak in luminescence intensity in all three groups 3–4 days after transplantation. Thereafter, the intensity of the AC-AC, AC-SY, and SY-SY groups decreased to values that were 1/160, 1/50, and 1/70 of the peak intensity, respectively. One explanation for these effects is that immediately following transplantation, the bioactivity of the transplanted cells was low due to the stress associated with transplantation. In addition, the amount of luciferin that could be delivered to the transplanted cells was also low due to potent edema in the transplanted region. With the gradual recovery of bioactivity and hemodynamic stability, the intensity of the light emission increased. However, as time passed, many transplanted cells died, resulting in the detection of luminescence from only the small fraction of transplanted cells that survived. When the same amount of luciferin was added *in vitro* to a luciferase-injected cell population, the luminescence intensity was nearly directly proportional to the number of cells. Therefore, the number of cells can be measured semiquantitatively. Although a strict comparison *in vivo* is difficult, we note that, in terms of measuring cell number from luminescence intensity, transplanted cells begin to die from the moment of transplantation, so by the time of peak intensity, the number of transplanted cells has decreased. As time passes, we infer that the number of cells decreases further, meaning that the detected luminescence is being emitted from surviving cells, which are much fewer in number than those present on the transplantation date. The detection of these remaining cells and their cell forms is a topic for future study.

Our ultimate goal is to develop cell sheets that, when transplanted, do not result in the conversion of hyaline cartilage within the regenerated cartilage tissue into fibrous tissue. In previous studies using Japanese white rabbits and pigs, we reported that using a combination of chondrocytes and synovial cells for cartilage regeneration prevented the conversion from hyaline cartilage into fibrous tissue [16,29].

Cell sheets offer a distinct advantage in the therapy of knee cartilage compared with cartilage chips and other methods that utilize a hard cartilage graft. The use of such a graft requires cumbersome techniques in that the graft is sutured or fixed via filler to the area around the damaged cartilage tissue. In addition, these techniques often give rise to graft attachment problems such as the generation of intra-articular loose bodies; by contrast, cell sheets tend to have fewer occurrences of these problems.

However, currently it is difficult to generate layered cell sheets comprised of human cartilage tissue because of the differences in the proliferative capability of human chondrocytes among individuals. In particular, human chondrocytes that are commercially available have already dedifferentiated to such an extent that their proliferative capability is low. The generation of cell sheets from such chondrocytes is oftentimes unsuccessful.

To facilitate the widespread use of human chondrocyte sheets generated through the co-culture method, and for augmenting prompt clinical use of these sheets, it is necessary to consider the safety and functionality of multilayered chondrocyte sheets. Further study in animal models and subsequent clinical trials are needed to clarify the utility and safety of chondrocyte sheets. At the same time, it is also necessary to evaluate the optimal source for fresh human chondrocytes, and to establish an effective means of storing chondrocyte sheets for allografting.

We believe that the development of regenerative therapy of osteoarthritis using cell sheets will provide a ready-made therapeutic strategy that, compared with conventional therapeutic strategies, is not substantially invasive, has high effectiveness, and can be used widely.

5. Conclusions

We used the BLI method to demonstrate that allografted cell sheets in knee joints survive for unexpectedly long periods of time. Both chondrocytes and synovial cells survived for an extended period of time at the transplanted site, thereby confirming the safety and utility of cell sheets in promoting cartilage regeneration.

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Articular Cartilage Regeneration Using Cell Sheet Technology

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ABSTRACT

Cartilage damage is typically treated by chondrocyte transplantation, mosaicplasty, or microfracture. Recent advances in tissue engineering have prompted research on techniques to repair articular cartilage damage using a variety of transplanted cells. We studied the repair and regeneration of cartilage damage using layered chondrocyte sheets prepared in a temperature-responsive culture dish. We previously reported achieving robust tissue repair when covering only the surface layer of partial-thickness defects with layered chondrocyte sheets in domestic rabbits. We also reported good Safranin O staining and integration with surrounding tissue in a minipig model of full-thickness cartilaginous defects in the knee joint. We have continued our studies using human chondrocytes obtained from patients under IRB approval, and have confirmed the safety and efficacy of chondrocyte sheets, and have submitted a report to the Ministry of Health, Labour, and Welfare in Japan. In 2011, the Ministry gave us approval to perform a clinical study of joint repair using cell sheets. We have just started implanting cell sheets in patients at Tokai University Hospital. Anat Rec, 297:36–43, 2014. © 2013 Wiley Periodicals, Inc.

Key words: cell sheet; articular cartilage; tissue engineering; regenerative medicine; temperature responsive culture dish

Articular cartilage is hyaline cartilage characterized by a compact collagen network and an extracellular matrix made up of proteoglycan, and is highly resistant to mechanical loads. However, articular cartilage possesses a limited capacity for complete repair (Paget, 1969). No meaningful repair of the cartilage occurs in partial-thickness lesions that are limited within the cartilage and leads to osteoarthritis (OA). OA, one of the most common joint diseases, is characterized by a slow degradation of cartilage for a long time. As society matures, as for much attention is being focused on OA prevention and countermeasures.

At present, treatments for osteochondral defects have included to date: microfracture (Steadman et al., 2001, 2002; Mithoefer et al., 2006), mosaicplasty (Hangody et al., 1997, 2001; Szerb et al., 2005), cell transplantation (Brittberg et al., 1994; Peterson et al., 2003; Zaslav et al., 2009; Moseley et al., 2010), and implantation of tissue-engineered cartilage with various scaffold materials (Buckwalter and Lohmander, 1994; Freed et al., 1994; Hunziker, 2002; Darling and Athanasiou, 2003; Marcacci et al., 2005) or without scaffold (Mainil-Varlet et al., 2001; Brehm et al., 2006; Park et al., 2006; Nagai et al., 2008a,b) have been developed to overcome this obstacle (Nagai et al.,

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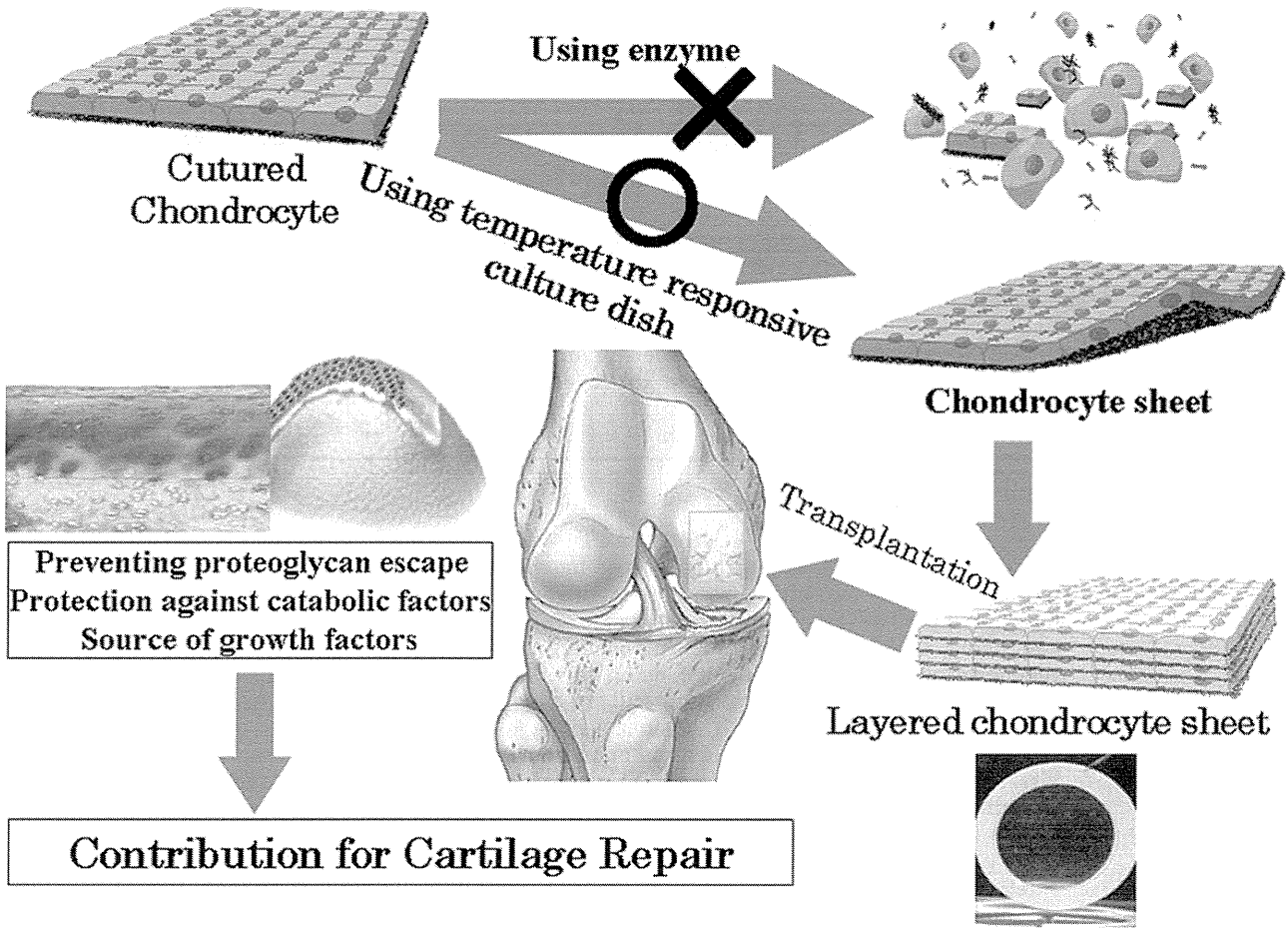

Fig. 1. Regenerative articular cartilage using layered chondrocyte sheets. Our goal is to contribute to the regenerative medicine of articular cartilage using layered chondrocyte sheets. These sheets display excellent adhesiveness and the ability to repair cartilage.

2010). Drilling and microfracture methods which promote to collect reparative cells from bone marrow are the methods that stimulate spontaneous healing (Buckwalter and Lohmander, 1994).

Autologous chondrocyte implantation (ACI), first reported by Brittberg et al. (1994), has been performed for over 20,000 patients worldwide. Normally, an osteochondral defect will induce the production of marrow-derived repair cells (Solchaga et al., 2000). Osteochondral defects are generally thought to be ultimately replaced by subchondral bone after infiltration by blood vessels during endochondral ossification of chondrocytes from multipotent, marrow-derived mesenchymal stem cells (MSCs) (Shapiro et al., 1993; Caplan et al., 1997). Nagai et al. (2008a,b) fabricated tissue-engineered cartilage without a scaffold and reported that "chondrocyte plates" were effective at repairing tissue in animal experiments. Among the established treatments, the preferred mode of treatment is ACI, which requires harvesting healthy articular cartilage cells from the patient and consequently causes donor site morbidity. Despite the promising results brought about by advances in tissue engineering, various limitations of ACI remain unsolved, including adverse events derived from periostium, a lengthy cell expansion period, and the cell delivery system. Improving ACI effectiveness and efficiency as a lifelong therapy for OA requires modifications of the current techniques and identification of other candidate cells for cartilage regeneration.

Cell sheet technology using temperature-responsive culture dishes was first reported by Okano et al., (1993) and is now used widely in corneal, myocardial, hepatic, and other regenerative medicine fields (Kushida et al., 2000; Harimoto et al., 2002; Nishida et al., 2004; Shimizu et al., 2006; Hamahashi et al., in press). Nishida et al. (2004) reported that corneal cell sheets fabricated using these dishes could adhere strongly to the cornea. Kushida et al. (2000) reported that the multilayered, three-dimensional tissue structures can be created without the need for scaffolds because cell sheets have intact extracellular matrix (ECM) and adhesion factors. Shimizu et al. (2006) reported that the maximum thickness of the fabricated rabbit myocardial cell sheet is three layers *in vitro* because thicker sheets receive inadequate nutrition. They also demonstrated that repetitive allografts of cell sheets *in vivo* could not increase the thickness by more than 1 mm in myocardial tissues (Hamahashi et al., 2012).

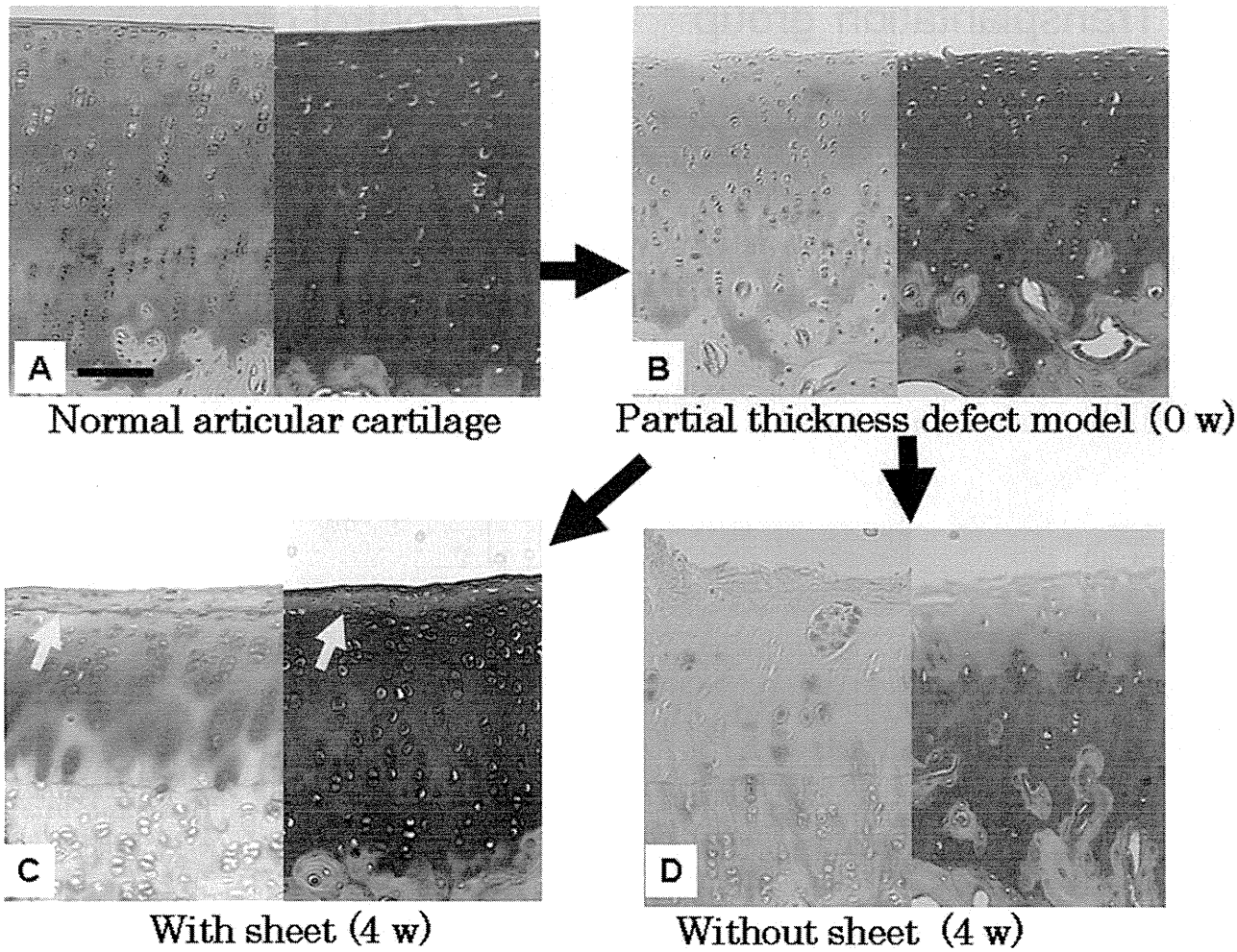

Fig. 2. Partial thickness defect model of rabbit. This is the therapeutic effect on a partial thickness defect. This partial-thickness defect model was produced by removing the surface layer of healthy cartilage. After 4weeks, in the absence of a grafted chondrocyte sheet, proteoglycans in the cartilage matrix flow out, causing cartilage degeneration that leads to OA. However, the matrix was maintained and degeneration failed to proceed in the transplantation group.

Using this innovational technology, we demonstrated chondrocytes sheets with a consistent cartilaginous phenotype and adhesive properties may lead to a new strategy for cartilage regeneration (Mitani et al., 2009) (Fig. 1).

CHONDROCYTE SHEET FOR PARTIAL THICKNESS DEFECT OF ARTICULAR CARTILAGE

The importance of the treatments and prophylaxes for OA is increasing due to the progressively aging society. However, we only have a few conservative therapies at this time, such as non-steroidal anti-inflammatory drugs (NSAIDs) administration and the injection of hyaluronan. Namely, there is still no means to prevent future exacerbations of cartilage degeneration (Kaneshiro et al., 2006). OA contains the partial-thickness defect of cartilage and the osteochondral defect (total thickness defect). Most regenerative therapies are aimed at the small osteochondral defect. Kaneshiro et al. (2006) reported the effects of the layered chondrocyte sheet on a partial-thickness defect in the rabbit. In that study, they allografted the three-layered chondrocyte sheet to repair the defect. This partial-thickness defect model was produced by removing the surface layer from healthy cartilage. The articular cartilage of the medial femoral condyle of Japanese White rabbits weighing about 3 kg was removed to a depth of less than 1 mm using a file to prepare a model of partial thickness cartilage damage. The damaged cartilage was covered with a three-layered chondrocyte sheet, which was stabilized with a nylon suture until the initial fixation was achieved. This was done in four knees of two rabbits as the transplantation group. At the same time, the articular cartilage of the medial femoral condyle was similarly filed, but not covered with a cell sheet, in four knees of the two rabbits (the control group) (Kaneshiro et al., 2006). After four weeks, in the absence of a grafted chondrocyte sheet, the proteoglycans in the cartilage

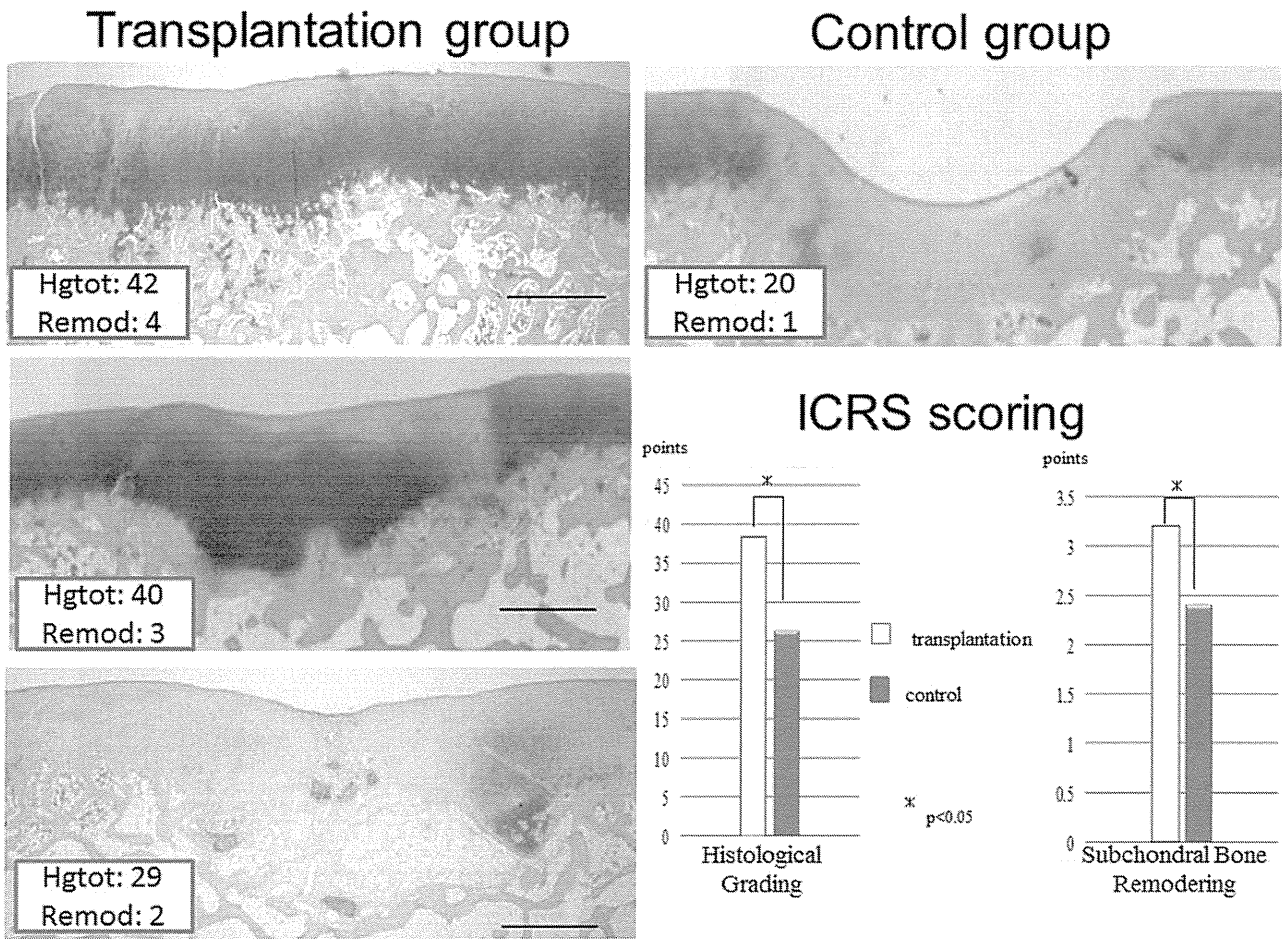

Fig. 3. Total thickness defect of minipig model. In most cases of the transplantation group, the ICRS histological grade was over 35 points, and the remodeling score was over 3 points. The mean histological grade and remodeling score were significantly higher in the transplantation group than in the control group.

matrix flow out, causing cartilage degeneration, which leads to OA. However, the matrix was maintained and degeneration failed to proceed in the transplantation group (Fig. 2). In this study, they confirmed that chondrocytes could be harvested as sheets and thus be made into multilayered "tissue" by culturing in temperature-responsive dishes and then be collected using a temperature recovery system.

Based on the above mentioned facts, they suggest that the use of bioengineered chondrocyte sheets may be potentially useful to treat partial thickness defects of articular cartilage. Because the chondrocyte sheets have good adhesion and barrier function which protect against intra-articular catabolic factors, supplying the growth factors (Kaneshiro et al., 2006).

CHONDROCYTE SHEET FOR TOTAL THICKNESS DEFECT (OSTEOCHONDRAL DEFECT) OF ARTICULAR CARTILAGE

Cartilage repair using synovial cell grafts has been carried out. Hunziker et al. have reported that synovial cells played an important role in the repair of the cartilage defects (Hunziker and Rosenberg, 1996), and Koga et al. (2008) have created osteochondral defects in rabbit knee joints and reported good results from grafts of synovium derived mesenchymal stem cells used in conjunction with periosteum. However, Ando et al. (2007) investigated repair of articular cartilage using chondrocytes and found that the superficial layers of the repaired tissue included fibrous tissue. Further investigation into osteochondral defects using larger animals has been carried out by Ebihara et al. (2012) using the minipig model (Fig. 3) and they have previously reported the efficacy of repairs using layered chondrocyte sheets. In order to solve the problem of fibrous tissue being included in the superficial layer in this experiment, we investigated the effects of treatment with layered chondrocyte sheets and synovial cell transplantation. Forty-eight white Japanese rabbits (female, age: 16–18 weeks, weighing: ~3 kg, with each group N = 4, six groups) were used in this study. In order to determine the effects of treatment, the following six groups were produced: (A) synovial cells (1.8×10^6 cells), (B) layered chondrocyte sheets (1.7×10^6 cells), (C) synovial cells (3.0×10^5 cells) + layered chondrocyte sheets, (D) synovial cells

(6.0×10^5 cells) + layered chondrocyte sheets, (E) synovial cells (1.2×10^6 cells) + layered chondrocyte sheets, (F) osteochondral defect. Layered chondrocyte sheets and synovial cells were transplanted, sacrificed 4 and 12 weeks postoperatively. An incapacitance tester (Linton) was used to find trends in the weight distribution ratio of the damaged limbs after surgery. Sections were stained with Safranin-O. Repair sites were evaluated using ICRS grading system. In Groups (A)–(E), the damaged limb weight distribution ratio had improved. The repair tissue stained positively with Safranin-O. Four and twelve weeks after surgery, Groups (A)–(E) exhibited significantly higher scores than Group (F), and Groups (D) and (E) exhibited significantly higher scores than Groups (A) and (B). This suggests the efficacy of combining layered chondrocyte sheets with synovial cells (Ito et al., 2012).

PREPARATION OF HUMAN CHONDROCYTES AND FABRICATION OF CELL SHEET INTO THREE LAYERED SHEETS

Human chondrocytes were obtained from the knee joints of young athletes who underwent anterior cruciate ligament reconstruction. Twenty-nine knees from 29 patients aged 14–49 years (21 males and 8 females) were used as the source of these cells. All subjects provided informed consent. The specimens were stored in basal medium (BM) containing Dulbecco's modified Eagle's medium/F12 (DMEM/F12; GIBCO, Invitrogen Corporation, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; GIBCO) and 50 μg/mL ascorbic acid (Wako Pure Chemical Industries, Ltd, Osaka, Japan) and 1% antibiotic–antimycotic mixture (ABAM; 10,000 U/mL penicillin G, 10,000 μg/mL streptomycin sulfate, and 25 μg/mL amphotericin B as Fungizone; GIBCO) until required for the next step. The cartilage samples were cut into small pieces. Thereafter, minced specimens were digested for 1 hr in BM containing 0.4% Pronase E (Kaken Pharmaceutical Inc., Tokyo, Japan), and for a further 4 hr in BM containing 0.016% Collagenase P (Roche Diagnostics GmbH, Mannheim, Germany). The digested cell suspension was passed through a cell strainer (BD Falcon™; BD Bioscience, Bedford, MA) with a pore size of 100 μm, and the isolated cells rinsed twice with chilled Dulbecco's phosphate-buffered saline (PBS; Dainippon Pharmaceutical Co., Osaka, Japan). The chondrocytes were then seeded into 500 cm^2 square dishes (245 mm × 245 mm; Corning Inc., Corning, NY) at a density of 10,000 cells/cm^2 and cultured in BM with 20% FBS (GIBCO) at 37°C in an atmosphere of 5% CO_2 and 95% air (Mitani et al., 2009).

To prepare the single-layer chondrocyte sheets, resuspended chondrocytes were seeded at a density of 10,000 cells/cm^2 in UpCell culture dishes (diameter: 35 mm, provided by CellSeed, Inc.). The seeded chondrocytes were cultured in BM adjusted to 20% FBS (GIBCO) at 37°C in an atmosphere of 5% CO_2 and 95% air. At 100% confluence, the cultured cells were harvested and prepared for gene expression analysis. To release confluent cells as a monolayer chondrocyte sheet from the UpCell temperature-responsive culture dishes, the dishes were removed from the incubator and let stand at about 25°C for 30 min. The culture medium was then removed from the dish, and the cell sheet harvested using polyvinylidene difluoride (PVDF) membrane as a supporting membrane. The lifted chondrocyte sheet edges promptly attached to the overlaid supporting membrane, and the cell sheet and PVDF membrane film were gently detached from the UpCell dish. Each cell sheet prepared as above was transferred onto another confluent chondrocyte sheet to fabricate multilayered sheets. Because the multilayered sheets spontaneously floated in culture medium, a 0.4-μm cell culture insert (Falcon, Becton Dickinson, NJ) was placed on top to prevent floating, and then culture of the sheets was continued for 1 week to obtain firm and perfect integration of the cells in the multilayer chondrocyte sheets (Mitani et al., 2009).

PROPERTIES OF HUMAN CHONDROCYTE SHEET AND HUMERAL FACTORS PRODUCED BY CHONDROCYTE SHEETS

The properties of the human chondrocyte sheets were investigated, including the expression and localization of SOX9, COL1, 2, 27, integrin α10, and fibronectin (Mitani et al., 2009). SOX9 activates COL2A1 in chondrocytes and directly regulates the Type II collagen gene *in vivo* (Wenke et al., 2006). Therefore, SOX9 is the one of the key regulators of chondrogenesis. Moreover, Jenkins et al. (2005) reported that the cartilage collagen gene, COL27A1, contains two enhancer elements that bind SOX9. Integrin α10 is specifically expressed in chondrocytes (Mitani et al., 2009). Chondrocytes, depending on the species and tissue origin, express a characteristic set of integrins, including receptors for collagen Type II (α1β1, α2β1, and α10β1), fibronectin (α5β1, αvβ3, and αvβ5), and laminin (α6β1). Among these receptors, integrin α10β1 is the major integrin mediating chondrocyte–collagen interactions in cartilage (Camper et al., 1998; Bengtsson et al., 2005). Mitani et al. (2009) reported that the significantly higher expressions of SOX9, integrin α10, and COL27 mRNA in the layered chondrocyte sheets revealed characteristics more closely resembling normal chondrocyte phenotype compared with chondrocytes in conventional monolayer culture.

Hamahashi et al. (in press) also investigated the humoral factors produced by layered chondrocyte sheets. Knee articular chondrocytes and synovial cells were harvested during total knee arthroplasty. After coculturing, the samples were divided into three groups: a monolayer, 7-day culture sheet group (Group M); a triple-layered, 7-day culture sheet group (Group L); and a monolayer culture group with a cell count identical to that of Group L (Group C). The secretion of collagen Type 1 (COL1), collagen Type 2 (COL2), matrix metalloproteinase-13 (MMP13), transforming growth factor-β (TGF-β), melanoma inhibitory activity (MIA), and prostaglandin E2 (PGE2) were measured by enzyme-linked immunosorbent assay. Layered chondrocyte sheets produced the most humoral factors. PGE2 expression declined over time in Group C but was significantly higher in Groups M and L. TGF-βexpression was lower in Group C but was significantly higher in Groups M and L ($P < 0.05$). Various humoral factors secreted from Layered chondrocyte sheets contains TGF-β and PGE2, which play an important role in cartilage repair and regeneration (Hamahashi et al., in press).

TGF-β stimulates chondrocytes to produce the extracellular matrices and also compete with the effects of

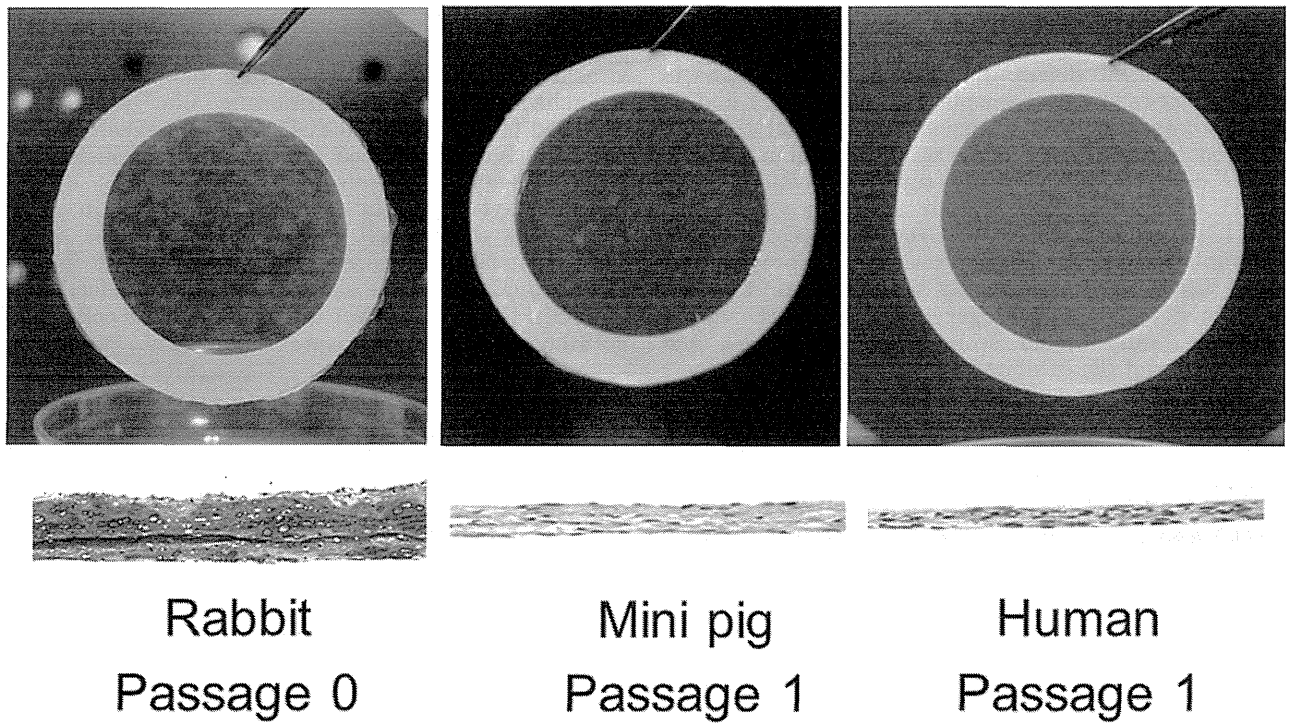

Fig. 4. Layered chondrocyte sheets. Layered chondrocyte sheets, even when composed of the same three layers, have different amounts of matrix accumulation and differing thicknesses depending on the species used. Especially, human chondrocyte sheet was quite thin and difficult to create on a regular schedule.

catabolic factors such as IL-1 (Blaney Davidson et al., 2006). The TGF-β signaling pathway is crucial for both maintaining cartilage homeostasis and preventing its disruption (Roman-Blas et al., 2007). Hamahashi et al. reported that TGF-β secretion levels were high in Group L in P0 and P1 but very low in P2, a pattern similar to that for COL2. PGE2 exerts pleiotropic effects in various tissues through EP1–4 receptors. In this study, they detected the higher secretory capacity for PGE2 in Group L and suggested that the implantation of layered chondrocyte sheets may have a therapeutic effect on partial-thickness cartilage defects. Aoyama et al. (2005) reported that EP2 is the main receptor expressed in articular cartilage, and the PGE2 signal through EP2 stimulates articular chondrocyte growth. And Nishitani et al. (2010) also reported that continuous inhibition of PGE2 accelerates the progression of OA. TGF-β and PGE2 also have an inhibitory effect on T cell proliferation (Di Nicola et al., 2002; Aggarwal and Pittenger, 2005). Hamahashi et al. (in press) also suggested that these inhibitory effects might be beneficial in counteracting immune rejection, such as graft-versus-host disease, suggesting one possible future clinical application.

ONGOING CLINICAL STUDY

Layered chondrocyte sheets, even when composed of the same three layers, accumulate different amounts of matrix and have differing thicknesses, depending on the species used (Fig. 4, cited with permission (a License Agreement of John Wiley and Sons provided by Copyright Clearance Center)). The human chondrocyte sheet is quite thin and difficult to create on a regular schedule. There are individual differences in human cell growth, so we coculture chondrocytes and synovial cells. This may mimic the intra-articular environment and provide an optimal environment for the preparation of chondrocyte sheets for tissue transplantation. This coculture system is particularly useful for reducing the culture period required.

We have confirmed the safety and efficacy of the chondrocyte sheet, and have submitted a report to the Ministry of Health, Labor and Welfare of Japan. In October 2011, the ministry approved a clinical study of joint repair using this cell sheet. We have just started the implantation of the cell sheet into patients at the Tokai University Hospital. We first use arthroscopy to diagnose the chondral defect precisely and to take cartilage and synovium from a nonbearing site. Each sample taken exceeds 1 g. Then we fabricate the layered cell sheet using the coculture method for three weeks at the Cell Processing Center (CPC). On the day of implantation surgery, after the cell sheet has been subjected to many kinds of tests, the layered cell sheets are carried to the operating room from the CPC. After the defect in the cartilage has been refreshed, we transplant the layered cell sheet. We implant the layered cell sheets using an open technique.

In 2008, the Government of Japan established the "Super Special Consortia" for Supporting the Development of Cutting-edge Medical Care. My colleagues and I belong to the "Project for the Realization of Regenerative

Medicine by Cell Sheets." We wish to develop the project as a hub for the rapid clinical application and commercialization of cell sheet technology in many areas of regenerative medicine. Tokai University is responsible for articular cartilage.

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Potential utility of cell sheets derived from the anterior cruciate ligament and synovium fabricated in temperature-responsive culture dishes

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Abstract: Development of tissue-engineered materials to treat anterior cruciate ligament (ACL) injury has been limited by the lack of phenotypic markers. We investigated the feasibility of inducing ACL regeneration using cell sheet technology based on the expression of tenomodulin (TNMD) as an early phenotypic marker of ligaments. ACL remnants, the synovium surrounding cruciate ligaments (SCL), the synovium surrounding the infrapatellar fat pads (SIF), and subcutaneous fat tissue (SCF) were obtained from patients undergoing ACL reconstruction or total knee arthroplasty. ACL cell sheets and SCL-derived cell sheets were fabricated successfully A three-dimensional bioengineered ACL was generated by combining triple-layered ACL cell sheets with a bioabsorbable mesh composite. Immunohistochemical examination showed that TNMD was expressed in human ACL fibers, triple-layered ACL cell sheets, ACL remnants, SCL, and SIF, but not in SCF. Real-time PCR showed that *TNMD* mRNA was expressed at substantially higher levels in the ACL, SCL, and SIF than in the SCF. These results suggest that TNMD is a specific marker of the human ACL and that ACL sheets have a phenotype similar to that of the ACL. The greater expression of TNMD in the SCL- and SIF- suggests that the synovium is a potential cell source for ACL regeneration. © 2013 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 00A:000–000, 2013.

Key Words: ECM, anterior cruciate ligament, synovial cell, cell sheet technology, tenomodulin

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INTRODUCTION

Human anterior cruciate ligaments (ACLs) are ruptured 200,000 times annually in the United States, involving medical costs of one billion dollars.[1] The ACL is an intra-articular ligament with limited healing capacity because its vascularization is poor. The current strategies for ACL repair involve reconstructive surgery with local autografts from the hamstring or patellar tendon, or allografts. Despite advances in reconstructive surgery and rehabilitation, obstacles to good outcomes remain. Graft donor site morbidity in ACL reconstruction based on autografts has been reported.[2–4] Prolonged weakness and muscular imbalance around the knee joint are risk factors for ACL reinjury, and allografts carry a risk of disease transmission by agents such as prions or human immunodeficiency virus.

In the 1980s, artificial ligaments became an attractive alternative to biological grafts. The initial enthusiasm surrounding their introduction stemmed from their lack of donor morbidity, abundant supply, considerable strength, ability to withstand immediate loading, and reduced need for postoperative rehabilitation. Synthetic grafts composed of materials such as carbon fibers, polypropylene, polyethylene terephthalate, and polyester are used either as prostheses or as biological ACL graft substitutes. However, every material has serious drawbacks, such as cross-infection, initiation of immune responses, breakage, debris dispersion

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leading to synovitis, chronic effusion, recurrent instability, or knee osteoarthritis.[5,6] Untreated ACL insufficiency can result in episodic instability, chondral and meniscal injury, and early osteoarthritis. Therefore, tissue-engineered materials are required to treat ACL injury. However, little is known about the genesis and configuration of tendons and ligaments, mostly because of the lack of early markers that distinguish regenerative ligaments and cicatricial tissues.

Cell sheet technology using temperature-responsive culture dishes was first reported by Okano et al.[7] Since then, this technology has been studied in the context of regenerative medicine for the cornea, heart, kidney, and liver.[8–12] Nishida et al.[10] reported that corneal cell sheets cultured in temperature-responsive culture dishes adhere strongly to the cornea without scaffolding or suturing. Kushida et al.[9] reported that fibronectin expression is preserved on the basal side of cell sheets cultured in temperature-responsive culture dishes. Because cell sheets can be harvested with the extracellular matrix (ECM) and adhesion factors, they can be layered using the natural adhesiveness of the basal side, allowing three-dimensional multilayer tissues to be constructed without a scaffold. We have investigated layered chondrocyte sheets for use in articular cartilage repair.[13–17] After considering the results of previous studies, we have begun a clinical study of chondrocyte sheets for articular regeneration, which was approved by the Ministry of Health, Labour and Welfare of Japan on October 3, 2011.

In a previous evaluation of potential cell sources for tissue-engineered ACL, Cooper et al.[18] reported that differentiated cells from the ACL are more suitable than are cells from the medial collateral ligament, Achilles tendon, or patellar tendon. Brune et al.[19] showed the potential utility of autologous cells extracted from biopsies of ruptured ACLs. The synovium has also attracted attention as a potential cell source for ligament tissue engineering.[20,21]

Tenomodulin (TNMD) is a type II transmembrane glycoprotein containing a C-terminal antiangiogenic domain and is expressed predominantly in tendons and ligaments.[22,23] We hypothesized that the presence of TnmD expression in fabricated cell sheets derived from ACL fibroblasts and synovial cells indicates that these sheets maintain some properties of ligaments.

In this study, we investigated the feasibility of inducing ACL regeneration using cell sheet technology based on the expression of TNMD as an early phenotypic marker of tendons and ligaments.

MATERIALS AND METHODS

Materials

This study complied with the Declaration of Helsinki and was approved by the Institutional Review Board for Clinical Research of Tokai University School of Medicine (ref. 04–056).

Human ACL remnants, the synovium surrounding the cruciate ligament (SCL), the synovium in the infrapatellar fat pads (SIF), and subcutaneous fat tissue (SCF) were obtained from 35 knee joints of 35 patients (22 men, 13 women; age range, 15–37 years) who underwent ACL reconstruction at Tokai University Hospital and Tokai University Oiso Hospital between December 2004 and October 2010. Human ACL fibers were obtained from five knee joints of five patients (two men, three women; age range, 68–79 years) who underwent total knee arthroplasty at Tokai University Oiso Hospital between April 2007 and March 2008. All the patients gave their written informed consent for the use of their tissues.

The specimens were stored in basal medium (BM) containing Dulbecco's modified Eagle's medium/F12 (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY), 50 μg/mL ascorbic acid (Wako Pure Chemical Industries, Osaka, Japan), and 1% antibiotic–antimycotic mixture (10,000 U/mL penicillin G, 10,000 μg/mL streptomycin sulfate, and 25 μg/mL amphotericin B as Fungizone [Gibco]) until they were required for the next step.

Preparation of ACL fibroblasts and synovial cells from the SCL, SIF, and SCF

The ACL remnants were removed from the injured site, cut into small pieces, and minced. The minced specimens of the ACL remnants, SCL, SIF, and SCF were digested for 0.5 h in BM containing 0.4% pronase E (Kaken Pharmaceutical Co., Tokyo, Japan) and for 2 h in BM containing 0.016% collagenase P (Roche, Mannheim, Germany). The digested cell suspension was passed through a cell strainer with a pore size of 100 μm, and the isolated cells were rinsed twice with chilled Dulbecco's phosphate-buffered saline (PBS).

Preparation of temperature-responsive culture dishes

The procedure used to prepare the temperature-responsive culture dishes (CellSeed, Tokyo, Japan) was as described previously.[7] In brief, *N*-isopropylacrylamide (IPAAm) monomer solution was spread onto commercial polystyrene tissue culture dishes. These dishes were then subjected to electron-beam irradiation, which induced the polymerization and covalent bonding of IPAAm to the dish surface. The poly-IPAAm-grafted dishes were rinsed with cold distilled water to remove any ungrafted IPAAm. The culture dishes were finally sterilized with ethylene oxide gas.

Preparation of single-layered ACL cell sheets and synovium-derived cell sheets

The ACL fibroblasts and cells prepared from the SCL and SIF were counted using a Bürker–Türk hemocytometer (Erma, Saitama, Japan). To prepare single-layered cell sheets, the resuspended cells were seeded at a density of 30,000 cells/cm^2 in UpCell temperature-responsive culture dishes (CellSeed). The seeded cells were cultured at 37°C in BM adjusted with 20% FBS (Gibco) in an atmosphere of 5% CO_2 and 95% air. To release the confluent cells as a monolayer sheet from the UpCell dishes, the dishes were removed from the incubator and left to stand at about 25°C for 30 min. The culture medium was then removed from the dishes, and the cell sheet was harvested using a polyvinylidene fluoride (PVDF) membrane (Durapore PVDF; Millipore, Billerica, MA) or CellShifter (CellSeed) as the supporting

membrane. The lifted cell sheet edges attached promptly to the overlain supporting membrane, and the cell sheet was detached gently from the UpCell dish.

Fabrication of triple-layered ACL cell sheets and synovium-derived cell sheets

Each single-layered cell sheet was transferred onto another confluent cell sheet to fabricate the multilayered cell sheets. Because the multilayered cell sheets floated spontaneously in the culture medium, a 0.4 μm cell culture insert (Falcon; Becton Dickinson, Franklin Lakes, NJ) was placed on top of the sheets to prevent them from floating, and sheet culture was continued for 1 week to obtain a firm tissue and the perfect integration of the cells in the multilayered cell sheets.

Immunohistochemistry

Frozen sections (30 mm × 24 mm × 5 mm) of triple-layered ACL cell sheets, normal ACL, ACL remnants, SIF, SCL, and SCF were prepared with OCT™ Compound (Sakura Finetechnical, Tokyo, Japan). The sections were washed in PBS and reacted at room temperature for 60 min with anti-human TnmD monoclonal antibody and serum (Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan), and purified with a MAbTrap™ Kit (Amersham Biosciences, Tokyo, Japan). The sections were washed in PBS and reacted with Alexa Fluor 488–avidin as the fluorescent secondary antibody. The sections were washed in purified water and then mounted with a water-soluble mounting medium (Vectashield® mounting medium with 4′,6-diamidino-2-phenylindole; Vector Laboratories, Burlingame, CA) to counterstain the DNA.

Primer design and real-time polymerase chain reaction (PCR)

All the oligonucleotide primer sets were designed based on published mRNA sequences. The expected amplicon lengths ranged from 70 bp to 200 bp. The entire coding region of the human *TNMD* cDNA (accession no. NM_022144) was cloned by 5′- and 3′-rapid amplification of cDNA ends using the following oligonucleotide primers: forward (5′-CCCAGCAGAAAAGCCTATTG-3′), reverse (5′-TTTTTCGTTGGCAGGAAAGT-3′).

Real-time PCR was performed in a SmartCycler system (Cepheid, Sunnyvale, CA) with the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and 1 μl of cDNA template in a final volume of 25 μl. The cDNA was amplified under the following cycling conditions: 35–45 amplification cycles of 95°C for 15 s and 60°C for 60 s. Changes in the SYBR Green fluorescence were monitored after every cycle. A melting curve analysis was performed as the temperature increased from 55 to 95°C at a rate of 0.5°C/s, with continuous fluorescence readings made at the end of the cycles to ensure that single PCR products were obtained. All the reactions were repeated in six separate PCR runs using RNA isolated from four sets of human samples. The results were evaluated using the SmartCycler software (Cepheid). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) primers were used to normalize the samples. To monitor the crossover contamination of the PCR, RNase-free water (Qiagen, Valencia, CA) was used in the RNA extraction and as the negative control. To ensure the quality of the data, a negative control was always included in each run.

Organ culture

A swine ACL partial-rupture model was prepared as follows. A resected swine knee joint was sprained manually to induce ACL elongation, and the partially ruptured ACL was excised with a scalpel. Triple-layered ACL cell sheets were then applied to this model. The cell sheets were cultured in BM adjusted with 20% FBS (Gibco) at 37°C in an atmosphere of 5% CO_2 and 95% air for 1 week. The specimens were then fixed *in situ* in 4% formaldehyde–glutaraldehyde in 0.1*M* sodium cacodylate solution at room temperature, embedded in paraffin, sectioned, and stained with toluidine blue for evaluation.

Fabrication of bioengineered ACL

The triple-layered ACL cell sheets were placed onto Vicryl® Mesh (a bioabsorbable material used widely in surgical treatment; Ethicon, Somerville, NJ) coated with fibrin glue. It was rolled up directly and tied at the edges to obtain a three-dimensional bioengineered tissue. These constructs were fixed *in situ* in 4% formaldehyde–glutaraldehyde in 0.1*M* sodium cacodylate solution at room temperature, embedded in paraffin wax, and sectioned perpendicularly (4.5 μm sections).

Statistical analysis

The real-time PCR results are expressed as the means ± standard errors of the means of six determinations. IBM SPSS Statistics version 17.0 (IBM SPSS, Chicago, IL) was used to perform the standard analysis of variance and Scheffé's *post hoc* test.

RESULTS

Manipulation of ACL cell sheets and SCL-derived cell sheets

The ACL cell sheets and SCL-derived cell sheets prepared as monolayer sheets were fabricated simply by reducing the temperature and without enzymatic digestion. The cells digested from the ACLs and SCLs were harvested as single contiguous cell sheets that retained the neighboring extracellular structure (Fig. 1). Triple-layered cell sheets were fabricated easily by layering ACL cell sheets and SCL-derived cell sheets with a supporting PVDF membrane or CellShifter, with or without fibrin glue.

Immunohistochemical analysis and TNMD *expression analysis*

TNMD was expressed in the human ACL fibers [Fig. 2(A)] and the cytoplasm of the triple-layered ACL cell sheets [Fig. 2(B)]. The rupture sites on the ACL remnants displayed inhomogeneous TNMD expression [Fig. 2(C)]. The protein was expressed clearly at the intact sites on the ACL remnants [Fig. 2(D)], SCL [Fig. 2(E)], and SIF [Fig. 2(F)], but not

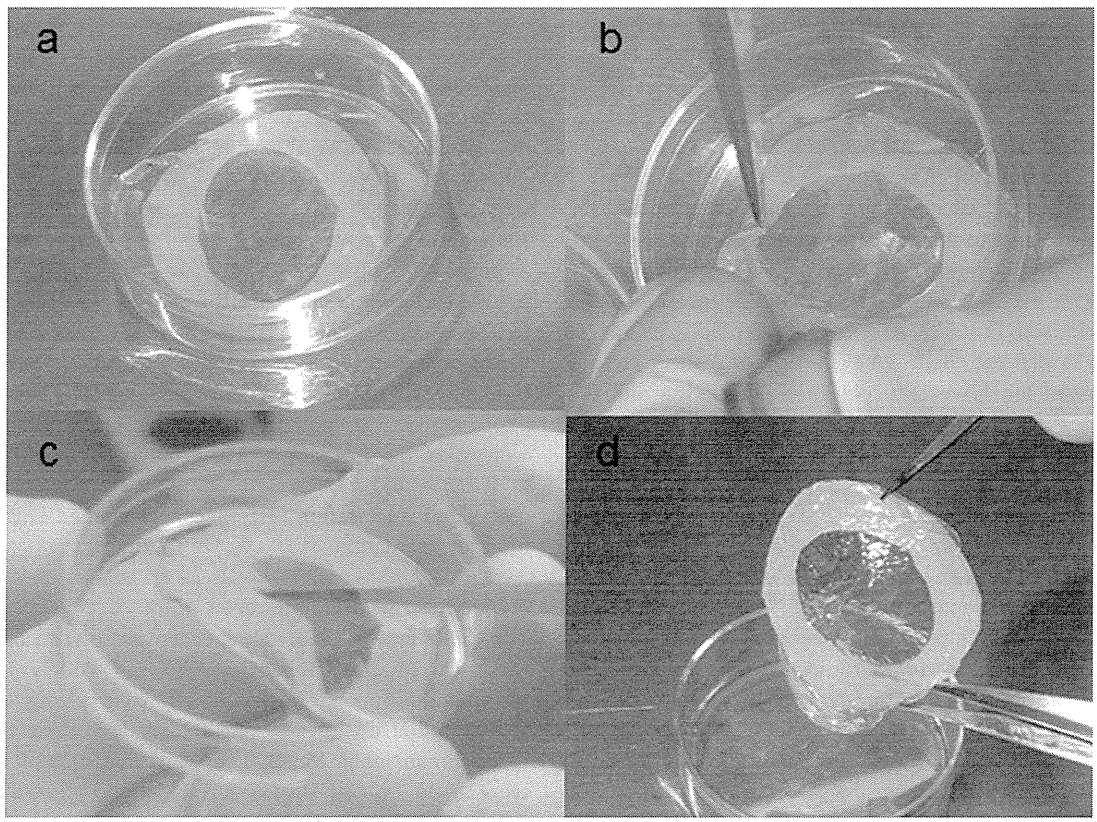

FIGURE 1. Fabrication of ACL cell sheets without enzymatic digestion. (a) A PVDF membrane is used as a supporting membrane and is coated with fibrin glue. (b) The lifted cell sheet edges attach promptly to the overlain supporting membrane. (c,d) The cell sheet is detached gently from the UpCell dish. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

in the SCF [Fig. 2(G)]. *TNMD* mRNA was expressed at significantly higher levels in the ACL, SCL, and SIF than in the SCF (Fig. 3).

Ex vivo *histological examination of triple-layered ACL cell sheets in the swine ACL partial-rupture model*

After the triple-layered ACL cell sheets and swine ACL partial-rupture model were organ cultured for 1 week, they remained attached to each other well [Fig. 4(A,B)]. Histological examination after toluidine blue staining confirmed that the cell sheets adhered directly to the swine ACL partial-rupture model [Fig. 4(C)].

Fabrication of three-dimensional bioengineered ACL

The triple-layered ACL cell sheets and Dexon mesh showed good adhesion [Fig. 5(A,B)]. Three-dimensional bioengineered tissues were obtained by rolling the cell sheets directly and tying the edges [Fig. 5(C)].

DISCUSSION

In tissue engineering, it is important to identify and fabricate cells and tissues that maintain their own phenotypes. The regeneration of tendons and ligaments has been limited by the lack of known specific markers that can distinguish regenerated tissues and indicate whether the phenotype has been maintained. Several recent studies have identified specific markers of tendons and ligaments. Schweitzer et al.[24] reported that the late expression of scleraxis is specific to the developing connective tissues that mediate the attachment of muscle to bone, including tendons, and to the ligaments that mediate the connections between bones. Asou et al.[25] reported that cells expressing scleraxis and Sox9 appear to be involved coordinately in the development of ligaments, tendons, and the skeleton. Shukunami et al.[23] reported that scleraxis positively regulates TNMD expression in a tendon-cell-lineage-dependent manner. Jelinsky et al.[26] reported that the genes encoding thrombospondin-4 and TNMD are highly expressed in rat tendons and ligaments, and are among the genes most specifically expressed in human tendons. In this study, we noted greater TNMD expression in intact human ACL fibers, but inhomogeneous expression at the rupture sites on ACL remnants, indicating that TNMD is a useful marker of intact ligaments in humans. The strong TNMD expression in the triple-layered ACL cell sheets also suggests that ACL cell sheets maintain some of the properties of ligaments. The ACL is a natural aggregation of fibers and includes a collagen-rich ECM. Therefore, ACL cell sheets fabricated with a rich ECM may help the ACL cell sheets maintain their ACL-like properties.

Brune et al.[19] suggested using autologous cells extracted from biopsies of ruptured ACLs as a cell source for tissue-engineered ACL constructs. Therefore, we fabricated cell

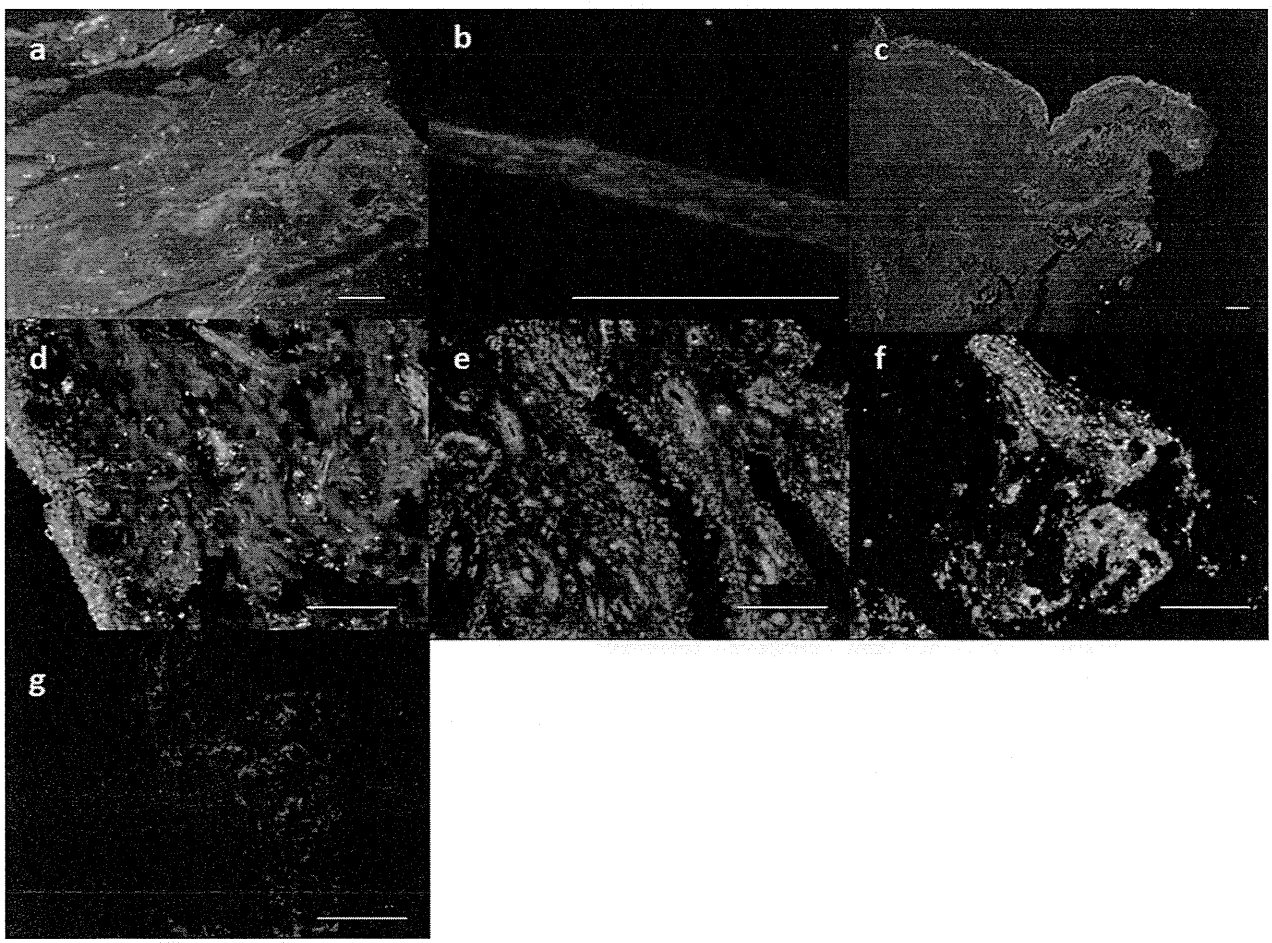

FIGURE 2. Immunohistochemical analysis. Fluorescence microscopy of ACL fibers (a), triple-layered ACL cell sheets (b), rupture sites on ACL remnants (c), intact sites on ACL remnants (d), SCL (e), SIF (f), and SCF (g). Scale bars: a, c–g: 100 μm; b: 10 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

sheets from ruptured ACLs. The strong TNMD expression in these sheets indicated that these ACL cell sheets have a phenotype similar to that of the ACL.

Sakaguchi et al.[21] reported the superiority of the synovium as a potential source of mesenchymal stem cells (MSCs) for cartilage regeneration. Morito et al.[27] noted an increase in synovial-fluid-derived MSCs in injured human ACLs. McGonagle and Jones[20] reported a potential role for synovial-fluid-derived MSCs in ligament regeneration. The strong expression of TNMD in the SCL and SIF and in SCL-derived cell sheets shows that the synovium is a potential cell source for ACL regeneration, consistent with the results of previous studies. The limitation of our method is that ACL cell sheets and three-dimensional bioengineered ACL do not have sufficient initial strength for their direct transplantation as regenerated ligaments. To acquire sufficient initial strength and the mature structures required to function as ligaments, these materials must be exposed to mechanical stress.

Anatomically, the ACL is divided into the anteromedial and posterolateral bundles, which help control the rotatory instability of the knee. When treating partial ACL rupture, Ochi et al.[28] reported that selective anteromedial or posterolateral augmentation using autogenous semitendinosus improved joint stability. In such cases, selective augmentation with ACL cell sheets using their own adhesiveness may improve joint stability without inducing donor-site morbidity.

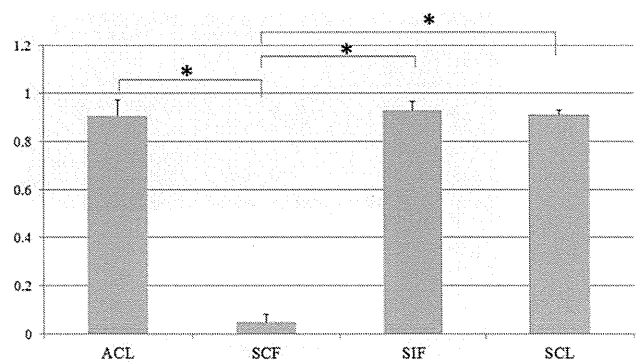

FIGURE 3. Relative expression of *TNMD* mRNA. *TNMD* mRNA expression was measured relative to that of *GAPDH*. The results were evaluated using SmartCycler II software. The error bars represent the standard errors of the means. $*p < 0.05$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

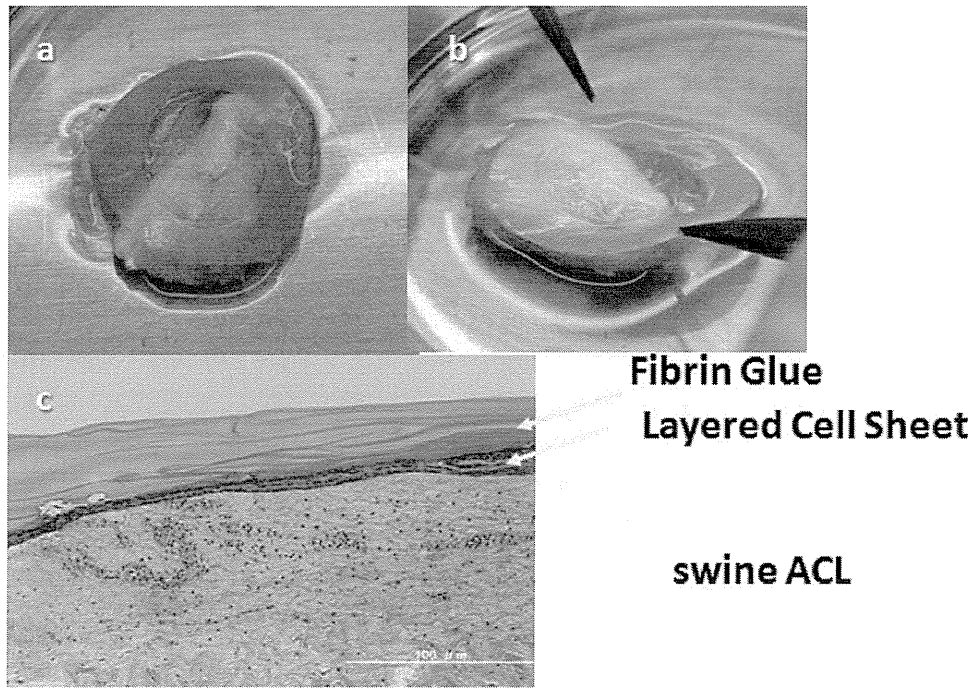

FIGURE 4. Application of ACL cell sheets to a swine ACL partial-rupture model. (a) A triple-layered ACL cell sheet was placed on the swine ACL partial-rupture model. (b) Adhesion of the sheet and partial-rupture model after 1 week of organ culture. (c) Histological examination after toluidine blue staining. Scale bar: 100 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

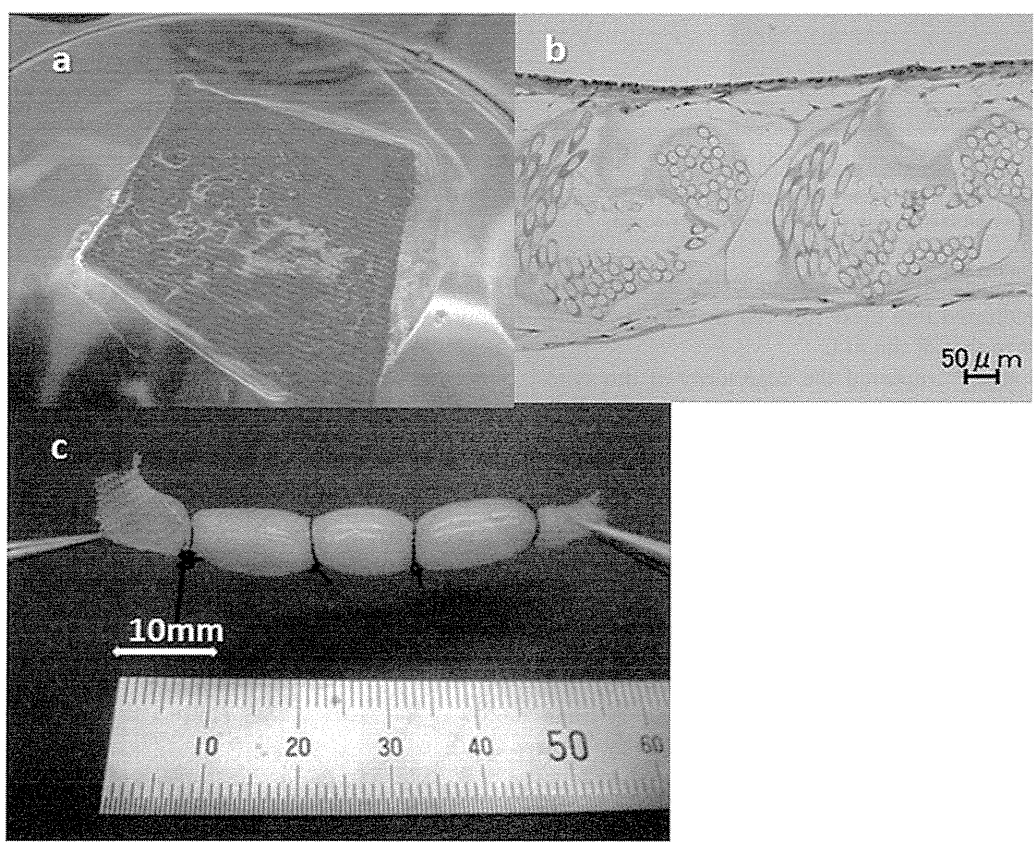

FIGURE 5. Fabrication of three-dimensional bioengineered tissue. (a) Triple-layered ACL cell sheets placed on Dexon mesh coated with fibrin glue. (b) Histological analysis of the combined cell sheets and mesh after toluidine blue staining. Scale bar: 50 μm. (c) The three-dimensional bioengineered tissue obtained by rolling the glued cell sheets and tying the edges. Scale bar: 10 mm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]