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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

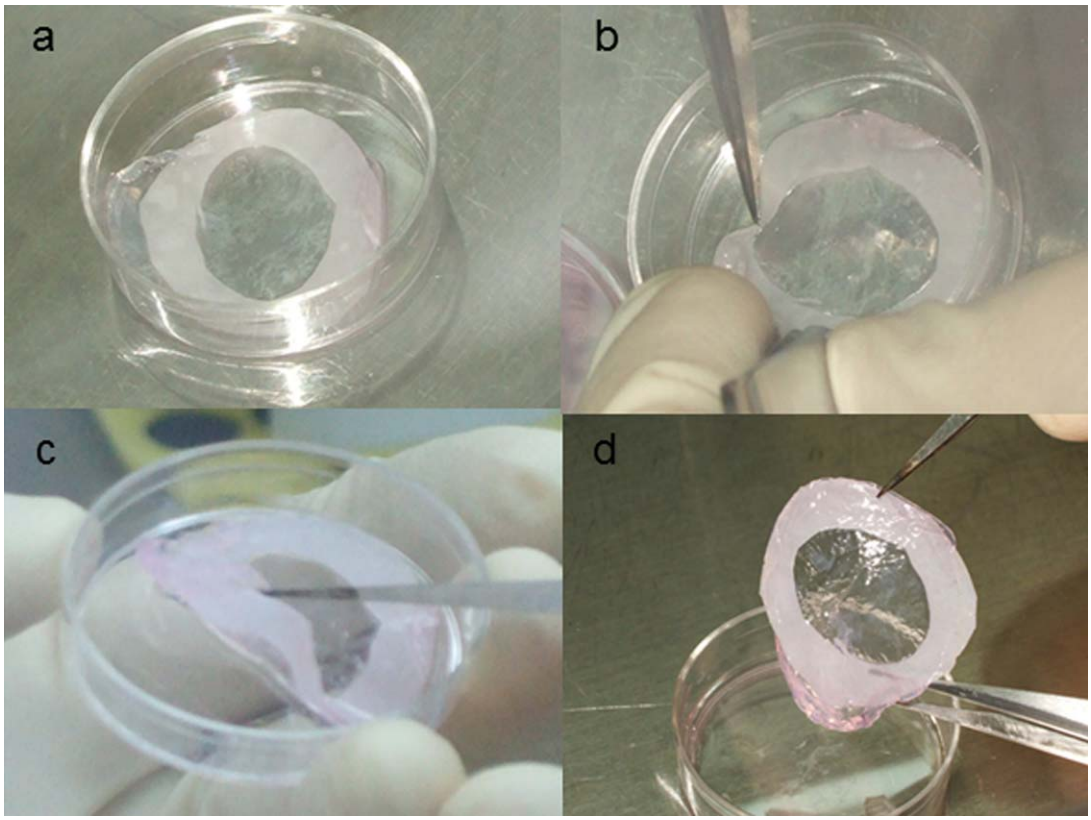

FIGURE 1. Fabrication of ACL cell sheets without enzymatic digestion. (a) A PVDF membrane is used as a supporting membrane and is coated with fibrin glue. (b) The lifted cell sheet edges attach promptly to the overlain supporting membrane. (c,d) The cell sheet is detached gently from the UpCell dish. [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

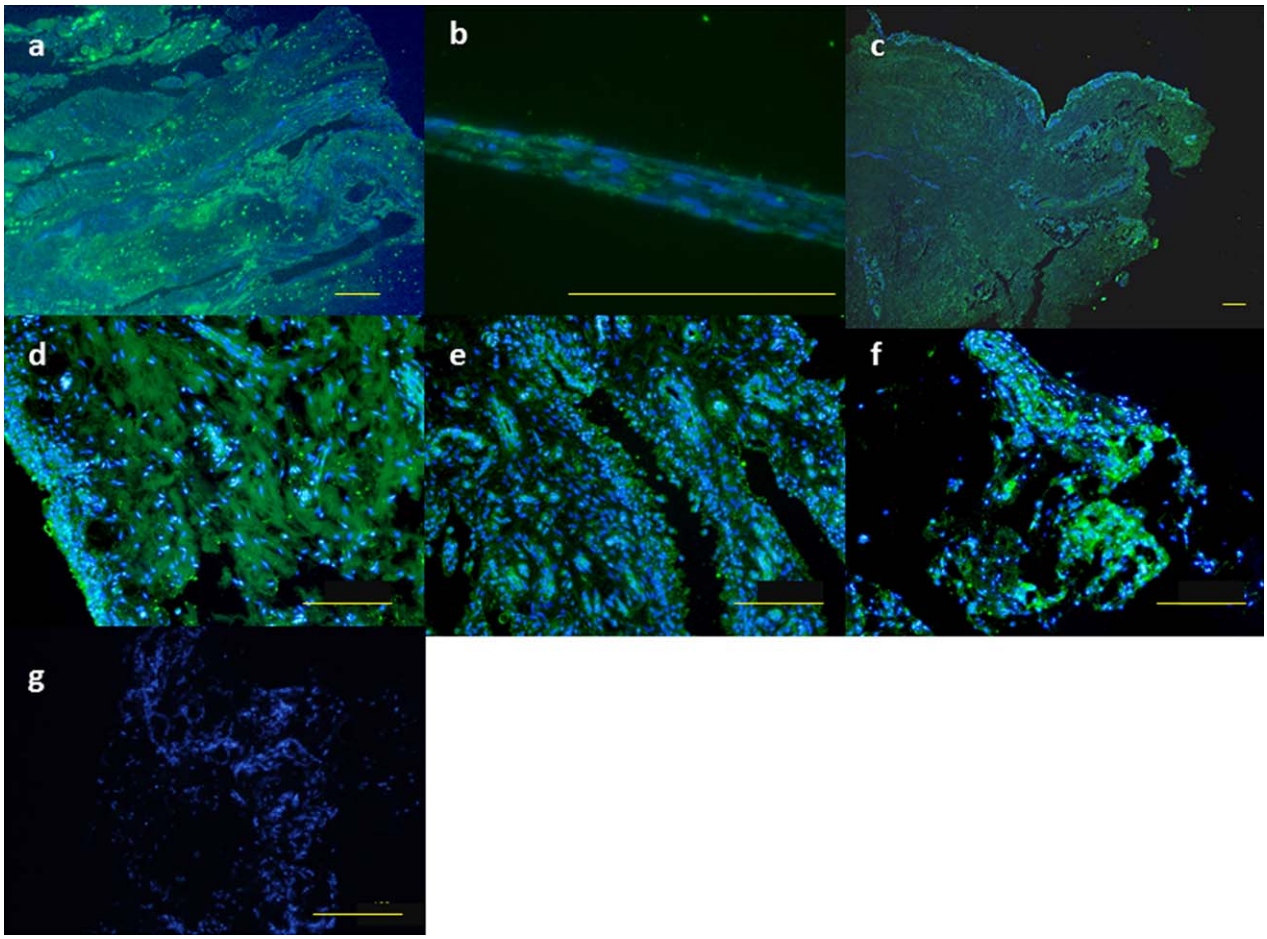

FIGURE 2. Immunohistochemical analysis. Fluorescence microscopy of ACL fibers (a), triple-layered ACL cell sheets (b), rupture sites on ACL remnants (c), intact sites on ACL remnants (d), SCL (e), SIF (f), and SCF (g). Scale bars: a, c–g: 100 μm; b: 10 μm. [Color figure can be viewed in the online issue,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

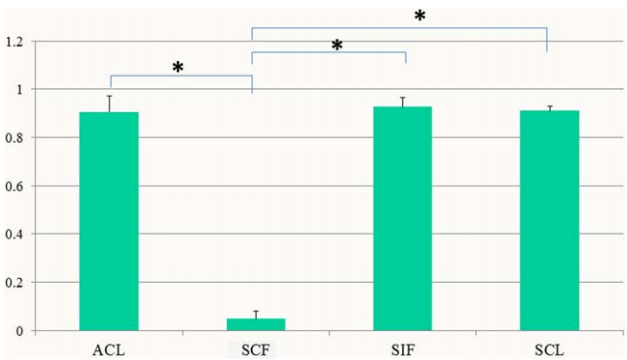

FIGURE 3. Relative expression of *TNMD* mRNA. *TNMD* mRNA expression was measured relative to that of *GAPDH*. The results were evaluated using SmartCycler II software. The error bars represent the standard errors of the means. $*p < 0.05$. [Color figure can be viewed in the online issue, which is available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

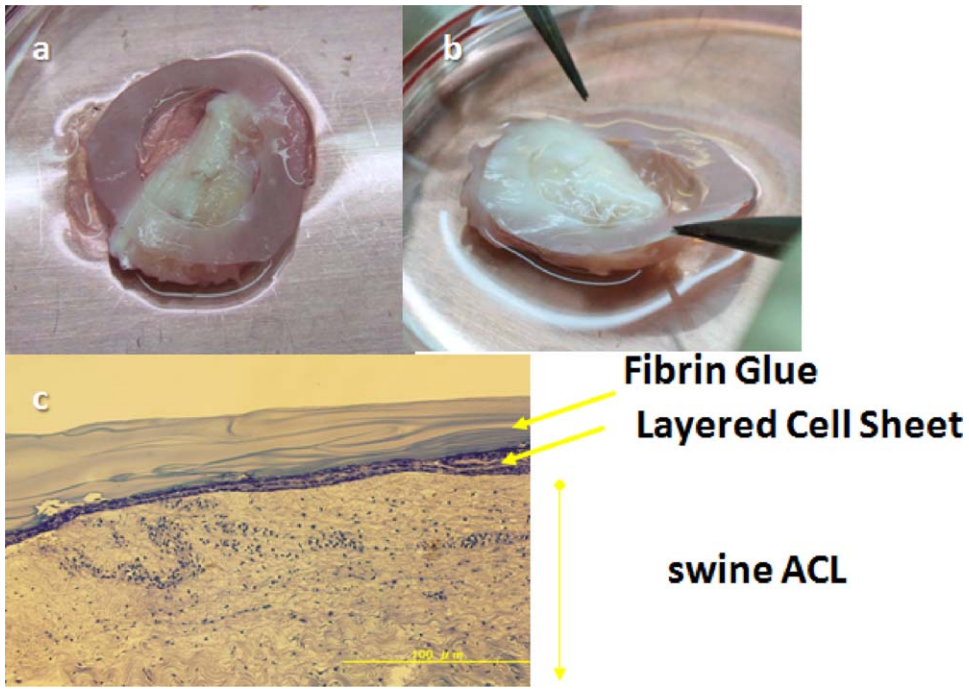

FIGURE 4. Application of ACL cell sheets to a swine ACL partial-rupture model. (a) A triple-layered ACL cell sheet was placed on the swine ACL partial-rupture model. (b) Adhesion of the sheet and partial-rupture model after 1 week of organ culture. (c) Histological examination after toluidine blue staining.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

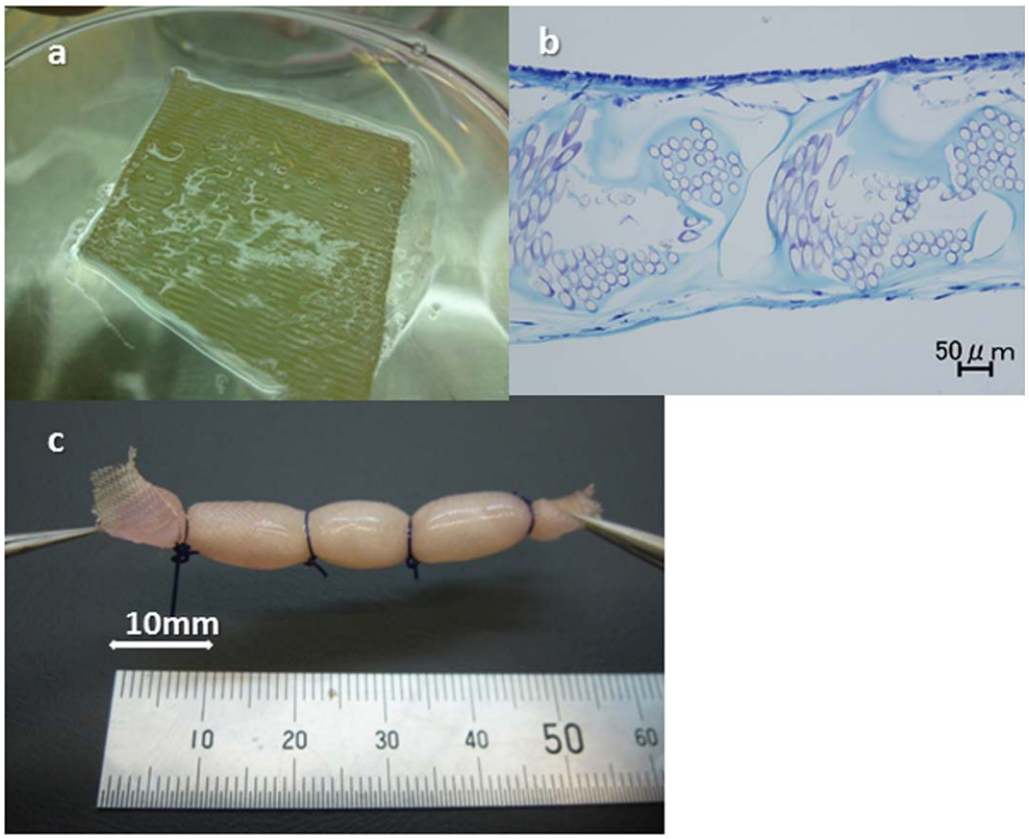

FIGURE 5. Fabrication of three-dimensional bioengineered tissue. (a) Triple-layered ACL cell sheets placed on Dexon mesh coated with fibrin glue. (b) Histological analysis of the combined cell sheets and mesh after toluidine blue staining. Scale bar: 50 μm. (c) The three-dimensional bioengineered tissue 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.]

Regardless of the potential value of these newly developed materials, further research is required to ensure their safe clinical application. Dexon mesh is a bioabsorbable material used widely in surgical treatments. The ability to repair incomplete ACL tears using self-adhesive ACL cell sheets and to fabricate three-dimensional bioengineered ACLs with a combination of cell sheets and materials currently used clinically represents considerable progress in ACL regeneration.

CONCLUSIONS

The elevated expression of TNMD in SCL-, SIF-, and SCF-derived cell sheets indicates that the synovium is a potential cell source for ACL regeneration. The presence of TNMD expression in fabricated cell sheets derived from ACL fibroblasts and synovial cells suggests that these sheets maintain some of the properties of ligaments. Cell sheet technology may be useful for fabricating tissue-engineered ligaments from these cells.

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