FULL PAPER

Using Cartilage Extracellular Matrix (CECM) Membrane to Enhance the Reparability of the Bone Marrow Stimulation Technique for Articular Cartilage Defect in Canine Model

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Bone marrow stimulation techniques (BSTs) are widely used in clinics to treat cartilage defects, but yet have a critical limitation from the loss of blood clots. In this work, a novel cartilage extracellular matrix (CECM) membrane is developed to protect blood clots after BSTs. The CECM membrane was made of ECM fabricated naturally by cultured porcine chondrocytes, and then decellularized and multi-layered to confer optimal mechanical strength. Highly compatible with cells, the CECM membrane did not show any cytotoxicity or immune responses in vivo. The CECM membrane was very thin (30–60 μm thick) and bendable, but had good tensile strength (85.64 N), suitable for protecting blood clots from leakage in rabbit cartilage defect. Moreover, the CECM membrane showed low but enough diffusion coefficient to allow delivery of small proteins in synovial fluid into the repaired tissue. In a beagle model, covering the cartilage defect with the CECM membrane after BST generated more hyaline cartilage-like tissues than the BST alone in histology and chemical analyses at 18 weeks. Its ICRS score was approximately 2.5 times higher than that of the BST alone. Therefore, the CECM membrane is proposed as a useful tool that can improve the outcome of BSTs to treat cartilage defects.

1. Introduction

Biomaterial has found extensive use, playing a crucial role as an implant by itself, drug delivery vehicle, cell delivery vehicle, or scaffold substrate in the tissue engineering. Many synthetic or natural biomaterials have been developed so far, and some studies tried to add functional structures or bioactive molecules to biomaterials for enhancing cell compatibility and tissue repair.[1–3] Among these approaches, use of extracellular matrix (ECM) materials is of particular interest. The ECM is thought to provide an optimal environment for differentiation of stem cells particularly into the cells of same origin as that of the ECM material. Thus, many studies attempted to employ tissue-specific ECM materials for the regeneration of the tissue.[4,5] Scaffolds using ECM materials have been manufactured from many different tissues such as dermis,

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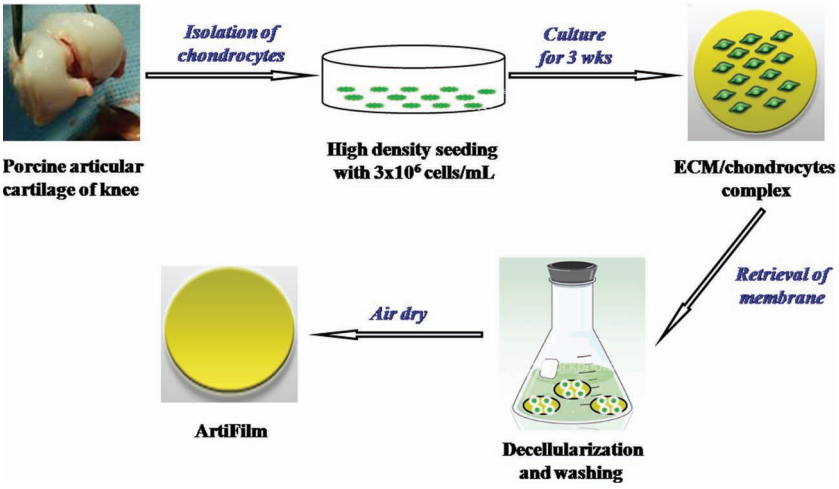

Figure 1. Manufacturing process of the cartilage extracellular matrix (CECM) membrane. Articular chondrocytes were isolated from porcine cartilage and subjected to monolayer culture at a high starting density (3×10^6 cells/mL) for 3 weeks in order to produce thin and firm membranes that were composed of a ECM/cell composite. The membranes were then decellularized and combined together in multiple layers in order to finally construct an CECM membrane of a thickness of 30–60 μm. Please refer to the Experimental Section for details.

small intestine, urinary bladder, heart, trachea, and lung and from a variety of species including human, porcine, bovine, and equine.[6–12]

We reported that an ECM scaffold derived from cultured porcine chondrocytes supported efficiently the proliferation of chondrocytes and formation of cartilage using chondrocytes or MSCs *in vitro* and in nude mice.[13–15] The ECM scaffold stimulated differentiation of bone marrow mesenchymal stem cells (MSCs) into chondrocytes and help differentiated tissues maintain well its phenotypes consequently proving it to be excellent in repairing and regenerating cartilage *in vivo*.[13] In addition, the cartilage defect in rabbits was well repaired and looked similar to that of normal cartilage after transplantation of an engineered cartilage produced using the ECM scaffold and rabbit chondrocytes. Such a cartilage specific biomaterial may stimulate the biological activity of chondrocytes and induce the differentiation of stem cells into chondrocytes, thereby is presumed to also increase the effectiveness of the surgical cartilage repair like bone marrow stimulation techniques (BSTs), that drain intrinsic stem cells to cartilage defect region.

BSTs have long been adopted to allow the migration of endogenous mesenchymal cells into the damaged area. What typically occurs with BSTs is that the subchondral bone in the cartilage defect under arthroscopic control is perforated or curetted to allow the bone marrow stem cells therein to move out and repair the defect site. Since Shapiro et al., Steadman et al., Breinan et al., Buckwalter et al. explained the origin of cells drained by BSTs, the repair was mediated wholly by proliferation and differentiation of mesenchymal cells of marrow.[16–19] The cells filled in the cartilage defect area start proliferating vigorously and thereafter slowly differentiate into chondrocytes. Here, the number of cells is very important. The number of cells that fill up the defect area of an immature animal was found to be similar to that of peripheral normal cartilage, which causes a significant impact on cartilage repair.[20] Therefore, undoubtedly, in order to reach those numbers, MSCs must have proliferated themselves. In this sense, preservation of MSCs drained from bone marrow will be very advantageous. Kramer et al., Dorotka et al., and Breinan et al. reported that cartilage healing was promoted by filling of the defect with matrix.[21–23] They suggested that holding the MSCs could be a crucial factor to enhancement of the regeneration potential. Mizuta *et al.* reported that the mobilization and accumulation of proliferative mesenchymal cells into the defect cavities correlated well with the capacity to subsequently induce cartilage repair. MSCs were differentiated into the chondrogenic lineage following the their proliferation.[24] The first evidence of synthesis of a cartilage extracellular matrix appeared at ten days.[16] Although type II collagen and aggrecan could be detected since 2 weeks and enhancement of type II collagen expression was observed at 6 weeks.[25] In spite that few reports was published regarding how differentiation of MSCs affects the cartilage repair after BSTs, MSCs being well differentiated surely lead better cartilage repair.

Although BSTs have shown poorly repaired tissue with mostly unstructured fibrocartilages and poor mechanical properties,[26,27] it is still considered the first line treatment for cartilage defects owing to its fairly easy procedure to perform and minimal invasiveness. Therefore, a new protocol in overcoming the limitations of BSTs and enhancing the regeneration of cartilaginous tissue in the defect is necessary, considering the number of cells and differentiation ability of progenitor cells.

We have developed a membrane form of the ECM material and applied it to covering the defect region after BSTs. The cartilage ECM (CECM) membrane was hypothesized to be able to preserve blood clots containing MSCs by not only acting as a physical barrier but also providing biological cues to enhance the repair of cartilage after BSTs. The physicochemical properties of the CECM membrane were first analyzed and its effect on repairing cartilage defect was evaluated in a beagle model.

2. Results

2.1. Gross Observation and EM images of CECM Membrane

The CECM membrane (**Figure 1**) looked translucent and stiff in gross observations (**Figure 2**A). In the SEM images, the cross-section of the CECM membrane showed a compact structure of multiple layers (Figure 2B, 1,500X), and its surface looked very smooth and compact (Figure 2C, 19X; and 2D, 220X) without any pores. In the TEM images with ultrahigh resolution (13,000X), the surface of the CECM membrane exhibited a rather rough morphology with many collagen fibers that were irregularly arranged and almost identically thick to those of normal cartilage (Figure 2E).

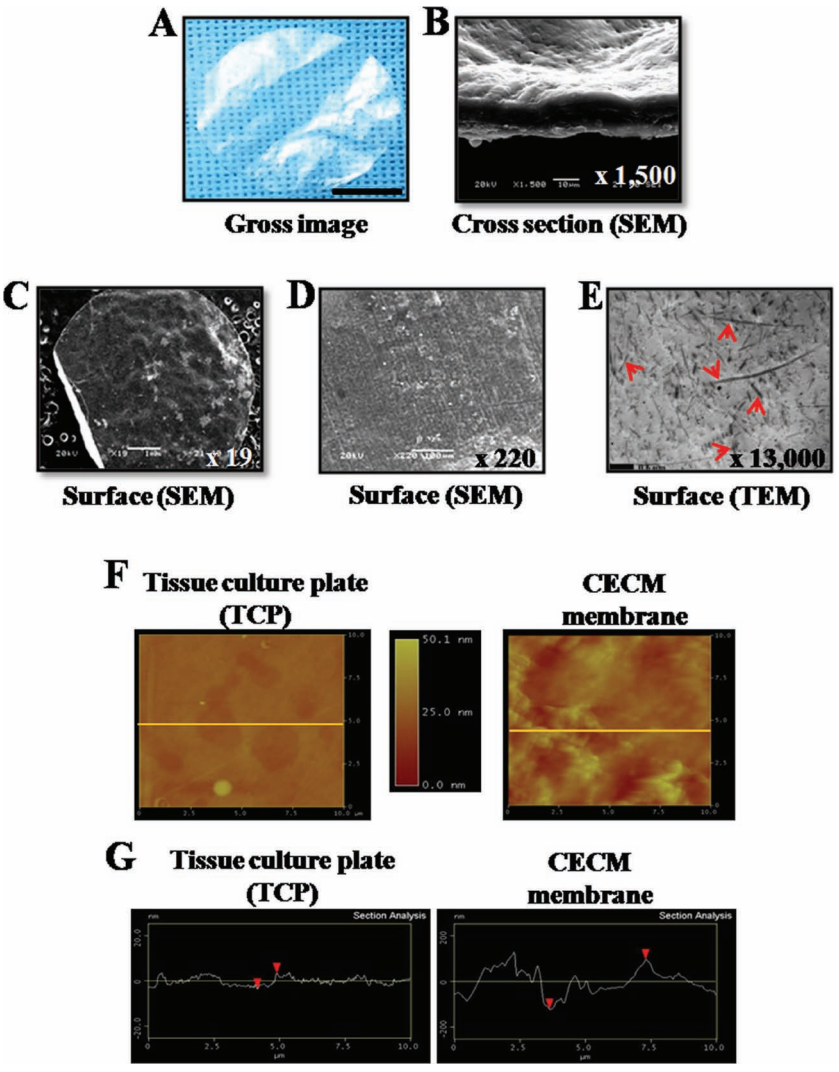

Figure 2. Morphology and surface analysis of the CECM membrane. (A) A gross image of the CECM membrane. Scale bar = 1 cm. (B) Sectional image of the CECM membrane obtained by scanning electron microscope (SEM; 1,500X). (C) Surface image of the CECM membrane obtained by SEM (19X). (D) Surface image of the CECM membrane obtained by SEM (220X). (E) Surface image of the CECM membrane obtained by transmission electron microscope (TEM; 13,000X). The arrows in E indicate collagen fiber-like structures. (F) The surface images of the CECM membrane and tissue culture plate (TCP) were obtained by atomic force microscopy (AFM). The depth of the surface area is indicated by the color gradient within the ranges of 0.0 (red)–50.1 (yellow) nm, as indicated in the middle. Cross lines in the images indicate the sites for topological analysis in G. (G) The surface topology of the CECM membrane and TCP along the cross lines are indicated in F. The 0 line indicates an average value of the topology, and 2 red arrowheads indicate the lowest and highest peaks for each sample. The roughness parameters of the CECM membrane and the TCP surfaces obtained by the topological analyses are presented in Table 2.

2.2. Physicochemical Properties of the CECM Membrane

The physicochemical properties of the CECM membrane are described in **Table 1**. The diameter of the CECM membrane was 28 ± 1 mm, which showed only small deviations because it was artificially cut to a circular shape in the final step. The tensile strength of the CECM membrane was 85.64 ± 6.49 N, which showed no significant variation within its thickness range. The water content of the CECM membrane was about 11 ± 3% of the total, which was much smaller than that of native cartilage (~80%). The collagen and sulfated GAG contents in the CECM membrane were 71 ± 3% and 16 ± 3%, respectively. Virtually no other proteins except collagen were detected in the CECM membrane (data not shown). For comparison, the collagen, sulfated GAG, and other protein contents in native cartilage are approximately 55%, 20%, and 25%, respectively.[31] The contact angle of the CECM membrane was measured at 68 ± 1°, which was slightly higher than 65.6 ± 1.5° of typical TCP.

Table 1. Physicochemical properties of CECM membrane.

Physical properties		Chemical properties	
Diameter	28 ± 1 mm	Water content	11 ± 3%
Thickness	30–60 μm	Composition (% dry weight)	Collagen: 82 ± 4%
			GAG: 18 ± 3%
Tensile Strength	85.64 ± 6.49 N	Contact angle	68 ± 1°

2.3. Surface Analysis of the CECM Membrane by AFM

When examined by AFM images, the surface of the CECM membrane was much rougher than that of typical TCP (Figure 2F and 2G). A quantitative analysis of the images using the Image J program revealed that the average roughness (R_a) of TCP was 1.564 nm with a standard deviation (R_{ms}) of 2.257 nm (**Table 2**). In contrast, the R_a and R_{ms} of the CECM membrane were 53.622 nm and 69.308 nm, respectively, which was much higher than that of TCP. The R_{max}, which was the difference between the highest and lowest heights, was 30.097 for TCP and 456.850 nm for the CECM membrane.

2.4. Cell Compatibility of the CECM Membrane

To evaluate the cell compatibility of the CECM membrane, the cell attachment and proliferation rates of rabbit chondrocytes on the CECM membrane were examined in comparison with those of TCP. The number of cells that were attached on the bottom was similar in both groups at 2 hour but was significantly increased on the TCP than on the CECM membrane at 4 and 8 hour (**Figure 3**A). At 24 hour, the values were similar in both groups, but the number was slightly more in the TCP (13,493 ± 356 cells/cm^2) than on the CECM membrane (12,031 ± 141 cells/cm^2), which

Table 2. Roughness parameters of CECM membrane by AFM images.

	R_a (nm)	R_{ms} (nm)	R_{max} (nm)
Tissue culture plate	1.564	2.257	30.097
CECM membrane	53.622	69.308	456.850

R_a is the arithmetic average of the height deviations from the center plane; R_{ms} is defined as the standard deviation of the height values (n = 3); R_{max} is the difference between the highest and lowest heights. The size of AFM images used for analysis was 10×10 mm^2.

was statistically significant ($p < 0.001$). The cell proliferation rate that was measured every day for up to 10 days was also higher on TCP, and the differences were significant at all time points after 4 days of culture ($p < 0.001$) (Figure 3B).

2.5. Permeability Property of the CECM Membrane

We found that about 77% of TGFβ3 accumulated for 96 hours after diffusion through the CECM membrane. Notably, the diffusion pattern was slow for the first 24 hours, and, afterwards, it became slightly faster for up to 96 hours. Based on these demonstrated patterns, the TGFβ3 diffusion coefficient was measured as 2.5×10^{-7}. This is an exceedingly low number, which indicates slow diffusion of the drug and demonstrates that the membrane is quite permeable (**Figure 4**B).

2.6. The Effect of the CECM Membrane on Fibrosis of Blood Clots After Bone Marrow Stimulation

In the gross and histological observations, the blood clots in the defect site felt soft and did not coagulate well, whereas those covered with the CECM membrane were a little hard and were observed to have fibrosis in progress (**Figure 5**). The characteristic red hue of blood had disappeared, and the area was covered with grayish-white fibrous tissues. The CECM membrane was observed to remain on the defect area at 1 week. No signs of inflammation or immune responses were observed in the histological examination.

2.7. Cartilage Repair in a Canine Model: Gross Observation and Safranin-O and Sirius Red Stain

Based on visual inspections performed 6 weeks after the surgery, both of the defect sites were packed with brown-colored tissues that, for the most part, had remained stained in hemoglobin unlike the surrounding cartilage tissues. Over time, however, the stained tissues were replaced with white glistening tissues that were similar to those found in the cartilage. The previously mentioned phenomenon showed no clear differences between the group with the CECM membrane and the one without it, however (**Figure 6**).

In the Safranin-O-stained images, all defects were mostly filled with fibrous tissues as early as 6 weeks. This finding persisted until 12 weeks in both the BST and the BST + CECM membrane groups (**Figure 7**A, 1L-6L and 1R-6R). After 18 weeks, however, the BST group did not exhibit metachromatic stains in the repaired tissues at the defect site, whereas the CECM membrane-covered group generally showed pale, metachromatic stains in the repaired tissues of 2 out of the 3 samples. The CECM group also revealed a metachromatic strain whose strength resembled that of normal cartilage in the deep layer (Figure 7A, 7R-9R versus 7L-9L). In the CECM group, chondrocytes formed mature lacunas and were perpendicularly disposed with subchondral bone. Besides, the formed GAGs were mainly observed in the deep layer (Figure 7A, 7R-9R). No signs of inflammation were observed. Overall, the CECM membrane group showed that the defect area was being more uniformly repaired compared to the BST group in terms of the height of the repaired cartilage and the staining strength.

The distribution and arrangement of collagen fibers were observed in the 18-week samples by Sirius red stain. As shown in Figure 7C, the collagen fibers were distributed irregularly and randomly in various directions in the BST group (a and b), while they were distributed more evenly and were aligned well vertically in the CECM membrane group (c and d).

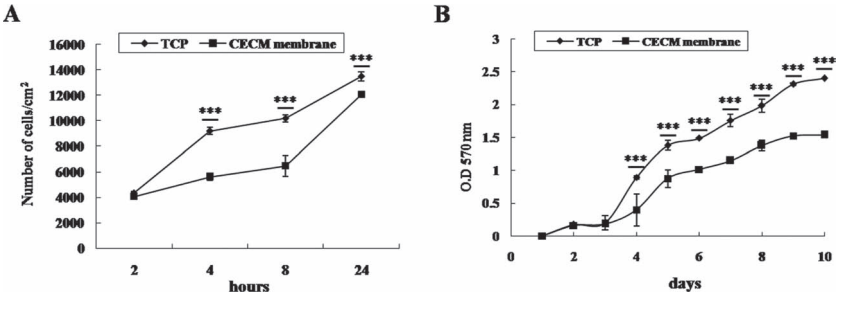

Figure 3. Cell compatibility of the CECM membrane. Rabbit chondrocytes (1.4×10^4 cells/cm^2) were seeded on the typical TCP or CECM membrane in 6-well plates. (A) The number of cells adhered to the unit area (1 cm^2) was measured at 2, 4, 8, and 24 hour before the first round of cell doubling was completed. (B) The proliferation rate of the cells in each group was measured by a MTT assay that was performed every day for up to 10 days. The absorbance at 570 nm is presented. Values are means ± standard error of the mean and are from 3 independent experiments (n = 3). Statistical significance was determined between TCP and CECM membrane groups at each time points. ***$p < 0.001$.

2.8. ICRS Scoring on Repaired Cartilage

The ICRS histological score did not change significantly over time until 12 weeks in both groups. The mean score of the BST group was higher than that of the CECM membrane group, but the difference was not statistically significant. A sharp increase in the score was observed at 18 weeks only in the CECM membrane group, which was increased approximately 3 times compared to the score of the BST group, while a slight decrease was seen in the BST group (Figure 7B, $p < 0.05$).

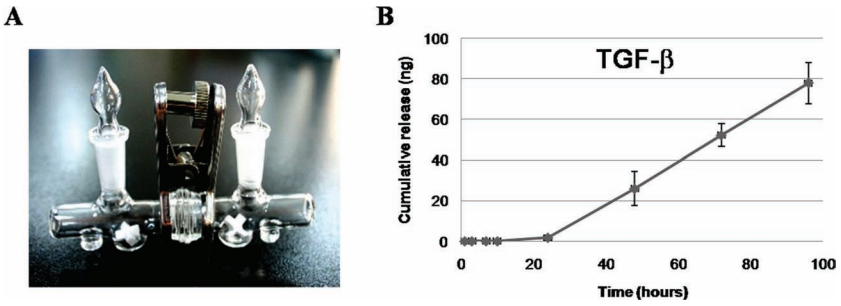

Figure 4. The cumulative release of loaded TGFβ3 that was transferred from the left chamber to the right through the CECM membrane.

2.9. Chemical Analysis of Repaired Tissue

The water (% total weight) and collagen (μg/mg dry weight) contents were not significantly different between the BST and CECM membrane groups over time (**Figure 8**A and 8D). However, the GAG contents gradually decreased in the BST group, while they gradually increased in the CECM membrane group, and they were 5 times larger in the CECM membrane group at 18 weeks (Figure 8B, $p < 0.001$). The DNA contents gradually decreased in both groups but showed a large variation in the BST group along with time (Figure 8C). The differences between the 2 groups were statistically significant only at 12 weeks ($p < 0.001$).

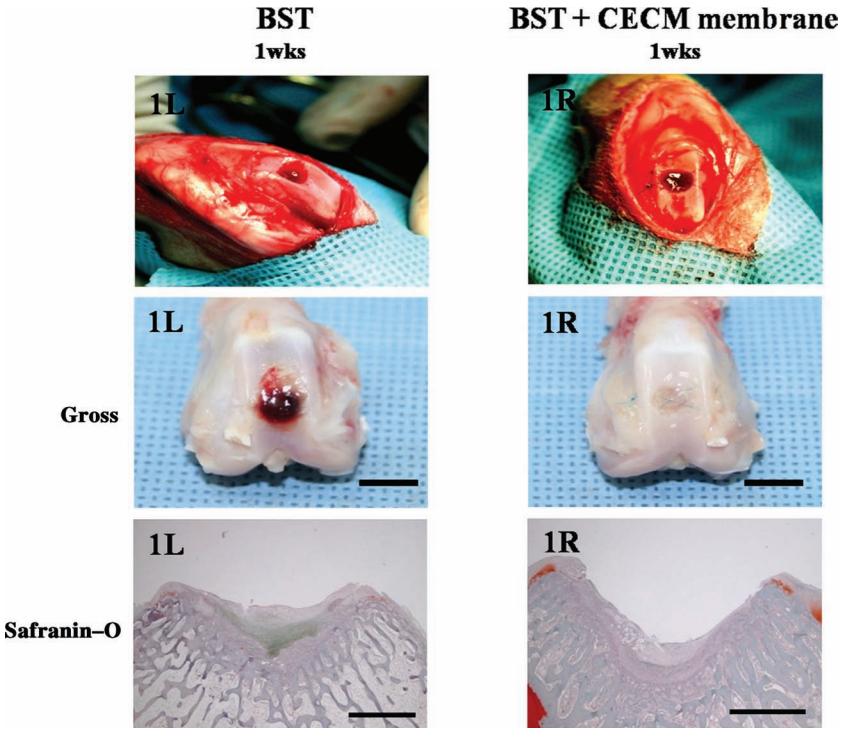

Figure 5. The application of the CECM membrane to the bone marrow stimulation technique (BST). The CECM membrane could be used to cover the defect area on the articular cartilage after surgery for BST, for example, BST arthroplasty (left; 1L). The CECM membrane was expected to stabilize the blood clots produced by BST and prevent the loss of mesenchymal stem cells (MSCs) from the defect area (right; 1R). The effect of the CECM membrane on the early tissue formation after BST is shown in a rabbit model. The cartilage defects were made in both knee joints in rabbits and they were treated with the microfracture arthroplasty as described in the Materials and Methods. The defect areas were then left uncovered in the left knees (BST alone; 1L) or covered with CECM membrane in the right knees (BST + CECM membrane, 1R). The gross morphology and the Safranin-O staining results at 1 week after treatment are presented as indicated (n = 3/group). Scale bars = 0.5 cm in gross images and 0.2 cm in Safranin-O images.

3. Discussion

The endogenous cell therapy of BSTs such as microfracture has long been applied to deliver cells into the defect area of cartilage. The brief biology of microfracture includes MSCs recruitment, attachment, and cell nesting. The retention of MSCs and their proliferation to fill up the defect should follow to regenerate the cartilage. The mobilization and accumulation of proliferative MSCs into the defect cavities correlated well with the capacity to induce cartilaginous repair subsequently.[24] Therefore, holding MSCs could be a crucial factor in enhance offing the regeneration potential of BSTs. The cartilage healing was also reportedly promoted by the filling of the defect with a matrix following BSTs.[21,22,23] Instead of using the collagen-based membrane in the previous report,[18] this study showed that cartilage defects covered with a cartilage CECM membrane after BSTs regenerated more hyaline cartilage-like tissues in the histology than those treated with BSTs alone. The improved outcome in the beagle model is attributable to the function of the CECM membrane to preserve better the blood clots and probably the MSCs therein. In the histology, the biomembrane did not exhibit therapeutic effect during the early stages of cartilage regeneration. Far from being effective, the material seemingly had a tendency to hamper the packing of the defect site at 6 and 12 weeks after the surgery. At 18 week after the operation, however, the repaired cartilage was observed to start to grow faster in the CECM membrane groups and exhibit a fairly noticeable increase in its hyaline-like characteristic. These findings could be supported by the report by Shapiro *et al.*[16] wherein the rabbit MSCs began differentiating into chondrocytes at 3 weeks after the surgery, then fair differentiation to chondrocyte took place, and eventually well-developed underlying cells of the repaired tissue become hypertrophic in 24 week after the operation. In the study with monkeys (Cynomolgus macaques), significant improvements in the extent and quality of cartilage repair were observed from 6 to 12 weeks after microfracture.[32] This study used beagles as an animal subject, yielding results similar to those summarized above.

Badylak reported that the implantation of biological ECM scaffold materials promotes

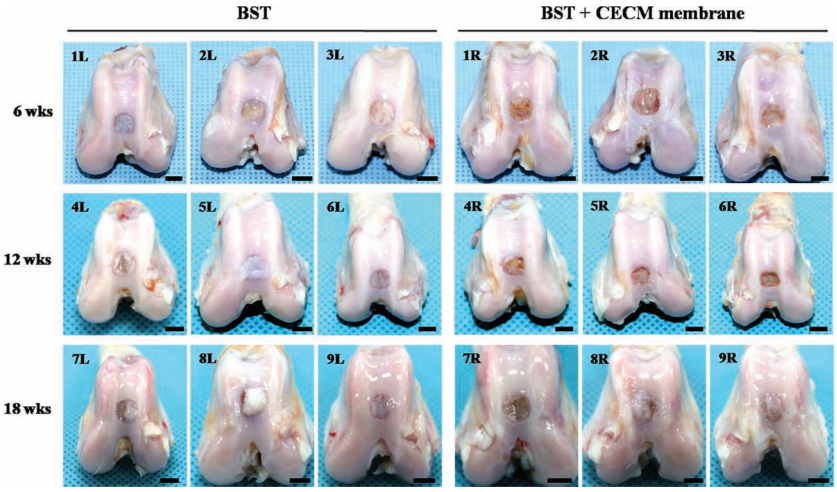

Figure 6. Gross observation of the cartilage defect at 6, 12, and 18 weeks after BST in a beagle model with or without CECM membrane application. The cartilage defect model and the BST were performed on both knee joints of beagles as in Figure 5 (n = 3/group). The defect areas were uncovered on the left (BST alone, L series) and covered with CECM membrane on the right knee joints (BST + CECM membrane, R series). The beagles were sacrificed at 6, 12, and 18 weeks after treatment. The gross images of all 3 samples per group are presented as indicated. The numbers indicate individual beagles, and the L or R indicates left or right knee joints. Scale bars = 0.5 cm.

site-specific constructive remodeling response. Following therapeutic placement and exposure to endogenous microenvironmental cues, the ECM scaffold degrades; it is then replaced by host cells and neo-ECM that can approximate the normal tissue architecture and function in that specific anatomical location.[33] Rich in native cartilage, type II collagen and sulfated GAGs have been previously shown to provide a favorable environment as scaffold materials for the proliferation of chondrocytes and chondrogenic differentiation of MSCs.[34,35] Moreover, in previous studies we have shown that a sponge-type version of the CECM membrane was highly efficient in cartilage tissue formation using rabbit chondrocytes and MSCs *in vitro* and *in vivo*, when compared with a mesh-type polyglycolic acid (PGA) scaffold with superior physicochemical properties.[13,15,36] In addition, the ECM scaffold not only strongly supports the chondrogenic differentiation of rabbit MSCs but also helps maintain its phenotype *in vivo*.[13] Even though this study did not prove if this environment was more favorable for tissue formation, the CECM membrane is assumed to provide more benefits for the repair of cartilage defects after BSTs as well, beyond serving simply as a physical barrier. The biggest weakness of this experiment lies in the lack of comparative results with other biomaterials. Breinan *et al.* reported collagen membrane,[18] whereas Hoemann *et al.* used chitosan-glycerol phosphate after performing microfracture for coverage of the cartilage defect.[37] Note, however, that those materials had some thickness and were inserted in the defect to absorb blood clots and consequently adsorb MSCs from the bone marrow. Therefore, they are considered to have a different cartilage repair mechanism compared with the membrane used in this study. No material suited for insertion in between the narrow joint while covering the area of cartilage defect could be found. Therefore, the relative superiority of the ECM biomaterial adopted in this study cannot be discussed.

Biomaterials derived from decellularized tissues and organs have been successfully used in tissue repair, because the xenogenic or allogenic cellular antigens were removed, while the structural and functional ECM molecules were well preserved.[38,39] Unlike the previous ECM biomaterials, the CECM membrane used in this study was made of ECM molecules released by cells (chondrocyte) during cultivation. To the best of our knowledge, this CECM membrane is the first material created by cell engineering. Since this CECM biomembrane has very similar chemical composition to natural cartilage, it is expected to reconstruct the characteristics of natural cartilage when implanted in the defect. Also, the cartilage is an extraordinary tissue with a unique immune-privileged property. Although the precise mechanism underlying such an extraordinary characteristic has yet to be explained, the ECM in the cartilage is believed to play a critical role in the relevant mechanism. Thus, the cartilage ECM secreted from chondrocytes is expected to have the same function.[40,41]

Implications on the surgical methods using the biomaterial scaffolds may carry more importance than that on the physiological strength. When applied to cover the cartilage defect area after BSTs, a scaffold should be thin enough to avoid hindering the sliding movement against the opposite cartilage and impeding the creation of blood clots from the subchondral bone by occupying the defect area. The CECM membrane has suitable physical properties as a barrier. Although only 30–60 μm in thickness, the CECM membrane possesses tensile strength of more than 85 N. In other words, the CECM membrane is thin and flexible, yet tough in nature, probably made possible by the complex ECM network naturally fabricated by chondrocytes.

Even in immunologically secure cartilage tissues, however, the cellular components can be notoriously immunogenic. Thus, ensuring a decellularization method and process is imperative. In general, the current methods used to decellularize and isolate ECMs include a combination of physical (freezing, pressure, sonication, agitation) and chemical (enzymes, detergents, acids, alkalines) treatments typically tailored to the tissue or organ of interest. Logically, each of these treatments affects the components retained within the extracellular matrix, possibly altering the mechanical and material properties, ultrastructure, ability to support growth and differentiation of cells *in vitro*, and host response following implantation.[42,43] This study used only chemical treatment with DNase and sodium dodesyl sulfate (SDS) solution, yet it succeeded in eliminating DNA from ECM beyond measurement. In retrospect, this was attributable to the fact that the cultured ECM, unlike natural tissues, could have had greater access to the chemical agents. Our approach of using only minimum amount of chemical detergents had presumably minimized the loss of functional components of the ECM. When present in sufficiently small quantities, neither DNA nor α-gal epitope has been associated with poor outcomes

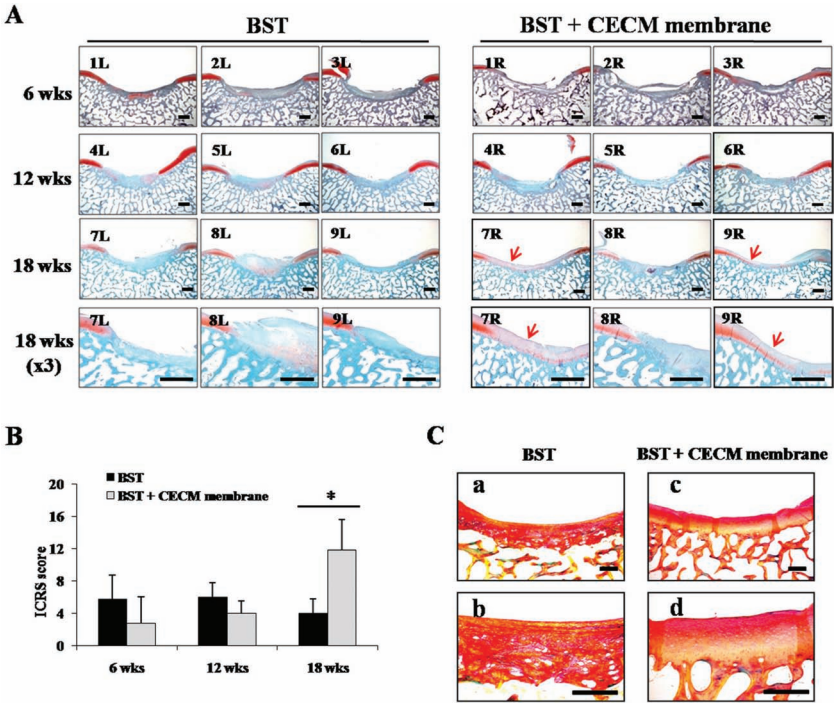

Figure 7. Safranin-O and Sirius red staining of samples. (A) The histological sections (4 μm) were prepared from all samples in Figure 6 and stained with Safranin-O in order to examine the amount of sulfated glycosaminoglycans (GAGs) in the newly formed tissues of the defect area. High magnification images of the regenerated tissues in the BST + CECM membrane group samples at 18 weeks are presented at the bottom. The accumulation of sulfated GAGs in the peripheral area of the defect area is indicated by arrows in the BST + CECM membrane group at 18 weeks. The labels in the figure are the same as in Figure 6. Scale bars = 0.5 mm. (B) The results of Safranin-O staining were evaluated by the International Cartilage Research Study histological scoring system. Values are means ± standard error of the mean from 3 individual samples (n = 3). The statistical significance was determined between the BST alone and the BST + CECM membrane groups at each time point. $*p < 0.05$. (C) The histological sections at 18 weeks were stained with Sirius red in order to examine the distribution and alignment of collagen fibers. Scale bars = 0.2 mm.

in vivo.[44,45] The non-traceable and small amount of DNA found in this study is therefore deemed unlikely to cause any harm in the host tissue. In fact, though it is originated in the porcine chondrocytes, the CECM membrane showed no clear signs of cytotoxicity or immune rejection during the histological observations following its implantation into the joint of beagles.

Another consideration to be made with cartilage is the lack of blood vessels. Such absence makes cartilage completely reliant on diffusion to secure the transfer of nutrients and growth factors as well as remove waste out of the tissues. Thus, the membrane to cover the repair site must meet this requirement. The CECM membrane is not completely waterproof; it is wettable in solution and permeable for the slow diffusion of soluble factors *in vitro*. Moreover, the CECM membrane showed a low but enough diffusion coefficient to deliver small molecules in the synovial fluid into the repaired tissue.

In this trial, we observed that covering the surgical site with a biomembrane caused fibrosis in the blood clots from the bone marrow to progress at a faster pace. This is presumably the consequence of blocking the disturbance of synovial fluid and the resulting expression of an inherent clotting cascade, which proved to be effective. With retaining MSCs inside blood clots as the primary goal of the biomembrane, an early onset of fibrosis similar to that found in this study can help maintain MSCs.

4. Conclusions

This study introduced a novel membrane-type biomaterial made of cartilage ECM that is derived from porcine chondrocytes. The CECM membrane successfully protected the blood clots produced by BSTs and enhanced the hyaline cartilage tissue formation in beagle models of cartilage defects. The CECM membrane not only has the appropriate biological properties that allow cell attachment and proliferation but also boasts suitable physical properties being semipermeable, biodegradable, and strong enough to protect blood clots. Considering that its components are similar to native cartilage and immune-privileged on implantation, these physicochemical properties of the CECM membrane are believed to make it a better choice not only for BSTs but also in other regenerative and reparative protocols of cartilage.

5. Experimental Section

Preparation of the CECM Membrane: Articular cartilages were obtained from the stifles of 2 to 3 week old pigs (Farmsco Co., Ltd., Seongnam, Korea).[15] The cartilage tissues were separated carefully from the other tissues, washed briefly with phosphate-buffered saline, and dissected into small pieces of approximately 1 mm^3. The cartilage pieces were then treated for 18 hour with 0.2% (wt/vol) collagenase (Worthington Biochemical Corp., Lakewood, NJ) in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Invitrogen Corporation, Grand Island, NY) supplemented with 5% newborn calf serum (NCS) (Thermo Scientific Hyclone, Logan, UT). Once the cartilage tissues were completely digested, the isolated chondrocytes were harvested by centrifugation at 1700 rpmfor 10 min. Cell pellets were washed twice with DMEM and seeded in 6-well tissue culture plates at a density of 7.5×10^5 cells per well. The chondrocytes were cultured in a monolayer with DMEM supplemented with 20% NCS, 1% antibiotic-antimyotic, and 5 μg/mL L-ascorbic acid. After 2 weeks, the cultured chondrocytes and ECM were collected by centrifugation at 2000 rpm. For decellularization, the cell-ECM complex was sequentially treated with 0.3% sodium dodecyl sulfate (SDS) solution 3 times for 2 hour each at RT, and 200 U DNase solution at 37°C for 2 hour. The decellularized ECM was finally washed with purified water for 30 min each and spread out to form a membrane. The resulting membranes were finally trimmed into a circular shape in order to produce a final product named ArtiFilm (**Figure 1**). The thickness of the CECM membrane ranged from 30–60 μm, which possibly depended on the thickness of the unit membranes (5–10 μm) before making multiple layers.

DNA Components in the CECM Membrane: DNA contents were determined by digesting dried samples in a papain solution (5 mM L-cysteine, 100 mM Na_2HPO_4, 5 mM EDTA, and 125 μg/mL papain type

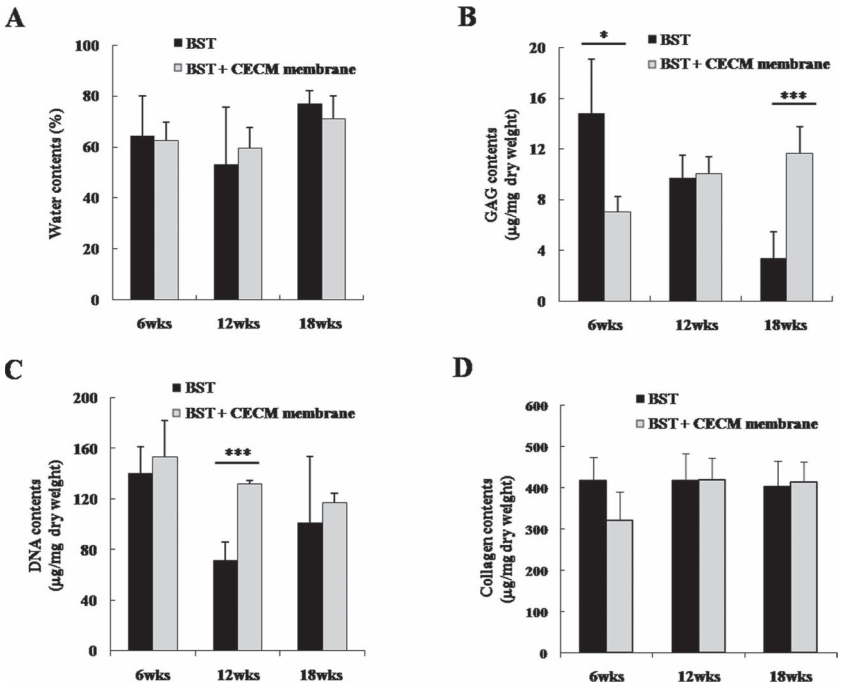

Figure 8. Chemical analysis of samples. The newly formed tissues were retrieved and digested with papain. The liquid samples were examined for the contents of water (A), sulfated GAGs (B), DNA (C), and collagen (D), as described in the Materials and Methods. The water content is presented as the percentage of water in the wet weight of samples (%). The results of the other assays are presented by the amount of each component in the dry weight of samples (μg/mg). Values are means ± standard error of the mean from 3 individual samples (n = 3). The statistical significance was determined between the BST and BST + CECM membrane groups. $*p < 0.05$ and $***p < 0.001$.

III (pH 6.4)) at 60 °C for 24 hours, which was followed by centrifugation at 12,000 × *g* for 10 min. The supernatant was used for the assays. The DNA content of the decellularized CECM membrane was measured using Quant-iT^{TM} dsDNA BR assay kit (Invitrogen Corporation; Eugene, OR).

Chemical Composition of the CECM Membrane: The CECM membrane was first digested in a papain solution (5 mM L-cysteine, 100 mM Na_2HPO_4 (pH 6.4), 5 mM EDTA, and 125 μg/mL papain type III) at 60 °C for 24 hours. The collagen content was measured by a hydroxyproline assay using bovine collagen of tracheal cartilage (Sigma-Aldrich Co., St Louis, MO) as a standard (0–10 μg/mL).[28] The glycosaminoglycan (GAG) contents were measured by a dimethylmethylene blue colorimetric assay using chondroitin sulfate from shark cartilage (Sigma-Aldrich Co.) as a standard.[29] The water content was determined based on the weight difference before and after drying the CECM membrane under 50–100 mmHg at 100 °C for 5 hour. The contents of collagen, sulfated GAGs, and water in the CECM membrane were described as a weight percentage of each element in the total weight (Table 1).

Physical Properties of the CECM Membrane: The physical properties consisting of diameter, thickness, and tensile strength of the CECM membrane were determined (Table 1). The diameter and thickness of the circle-shaped CECM membrane were measured using a caliper rule and a thickness-measuring instrument (ID-S1128; Mitutoyo Corporation, Kanagawa, Japan), respectively. The tensile strength of the CECM membrane was measured using a Universal Testing Machine (H5K-T; H.T.E., Salfords, England). Briefly, the 1 × 3 mm^2 specimens ($n = 5$) were placed vertically on a 500 N load cell and pulled at a speed of 10 mm/min. A break load was obtained from the load-displacement curve, and tensile strengths were then calculated.

Electron Microscope (EM) Imaging: The surface and sectional images of the CECM membrane were observed by scanning electron microscope (SEM) at 15 kV (Hitachi S-4700; Hitachi High-Technologies Corporation, Tokyo, Japan), and the collagen fiber arrangement was evaluated by transmission electron microscope (TEM) at 100 kV (Hitachi H-7650; Hitachi High-Technologies Corporation).

Atomic Force Microscope (AFM) Analysis: The surface of the tissue culture plate (TCP) and CECM membranes was analyzed using an AFM (MultiMode; Veeco Instruments, Inc., Plainview, NY) under a taping mode. The surface roughness of the samples was determined from the AFM images of 10 × 10 μm^2 using Image J image software. Image J is a public domain software program (http://rsbweb.nih.gov/ij/). Filament contours were traced and exported as XY-coordinate sets by using either the Freehand Linetool or a skeleton algorithm implemented in Image J.

Cell Compatibility of the CECM Membrane: Rabbit chondrocytes were isolated from the articular cartilages of 2 week old male New Zealand white rabbits (Joong Ang Experimental Animal Center; Seoul, Korea), as described above for the porcine chondrocytes. The isolated chondrocytes were cultured at an initial density of 1.0 × 10^6 cells per 150-mm dish. The chondrocytes at passage 2 were seeded on 6-well plates with or without an CECM membrane precoating at a density of 1.4 × 10^4 cells/cm^2. To examine the early attachment rate of chondrocytes, the unattached cells in the medium were collected and counted by Trypan blue staining at 2, 4, 8, and 24 hours after seeding. The proliferation rate of chondrocytes was measured by a MTT assay (Sigma-Aldrich Co.) every day until 10 days after seeding.

Permeability Property of the CECM Membrane: To confirm the permeability of the CECM membrane, we loaded 100 ng of TGFβ3 on one side of the diffusion cell chamber (Figure 4A) and measured the amount of drug released on the other side with the CECM membrane in the center for 1, 3, 7, 10, 24, 48, 72, and 96 hours, and then the release patterns were analyzed using ELISA kits (R&D system; MN, USA) in order to measure the diffusion coefficient of TGFβ3.

Effect of CECM Membrane on Fibrosis of Blood Clot: The cartilage defects of rabbit stifles were left alone or covered with CECM membrane after BST, which was conducted as described above, in 'Cartilage defect model and bone marrow stimulation technique' section. After 1 week of surgery, the specimens were evaluated by gross and histological observation.

Cartilage Defect Model and Bone Marrow Stimulation Technique: Male beagles (12–15 mo.; KyeRyong Science Co., Ltd., Daejeon, Korea) were used. The experimental animals were anesthetized with zoletil and lumpun (ratio, 3:1). An arthrotomy was made through a midline longitudinal incision on the medial parapatellar of both knee joints with the patella dislocated laterally to expose the femoral patellar groove. Full thickness chondral defects were created that were 5 mm in diameter on the trochlear groove of both knees using a biopsy punch. The defects were surgically treated with the BST. Briefly, subchondral cortical bone was partially removed by curet and then left uncovered in the left knee as a control group (BST group) or covered with CECM membrane in the right knee (CECM membrane group). After the BST, CECM membrane that was 6 mm in radius was attached to the surgical region and sutured in a criss-cross manner (Figure 5, 1R). The experimental protocol was approved by the Institutional Animal Experiment Committee of described Ajou University School of Medicine.

Histological Evaluations of Repaired Cartilage: The effect of the CECM membrane on the cartilage repair after BST was evaluated in the canine model of cartilage defects. A cartilage defect was made on both knees

of beagle dogs, and one knee was covered with an CECM membrane after the BST. The repaired cartilage was observed in the uncovered control (BST) and CECM membrane (BST + CECM membrane) groups at 6, 12, and 18 weeks. The specimens were decalcified by 5% nitric acid and then embedded in paraffin wax. Coronal sections that were 4 μm in thickness were prepared and stained with Safranin-O or Sirius red staining, as described previously.[14] The Safranin-O images were evaluated quantitatively by a modified version of the International Cartilage Research Society (ICRS) grading scale, which consists of 7 categories and which assigns a score ranging from 0 to 18 points.[30]

Chemical Analyses: The repaired tissues in the defect area were carefully removed using curets. The retrieved tissues were dried at 37 °C for 48 hours. The water content of the samples were determined by the percentage of water (wet weight–dry weight) in the wet weight of the samples, as described above, in 'Chemical composition of the CECM membrane' section. The dried tissues were digested in the papain solution and the sulfated GAGs and collagen contents were determined, as described above, in 'Chemical composition of the CECM membrane' section. The DNA contents of the samples were measured using Quant-iT™ dsDNA BR assay kit (Invitrogen Corporation).

Statistical Analysis: The statistical analyses of the experimental data were performed with one-way analysis of variance for multiple comparisons and Student's *t*-test (two-tail) for pair-wise comparisons. Differences were considered statistically significant if p values were less than 0.05.

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