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Skeletal Myoblast Cell Sheet Implantation Ameliorates Both Systolic and Diastolic Cardiac Performance in Canine Dilated Cardiomyopathy Model

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Background. Improving both systolic and diastolic function may be the most important factor in treating heart failure. In this study, we hypothesized that cell-sheet transplantation could improve these function in the damaged heart. **Methods.** We generated a dilated cardiomyopathy model in beagles by continuous ventricle pacing at 240 beats per minute. After 4 weeks, the beagles underwent skeletal myoblast cell sheet transplantation (SMCST) or a sham operation, and rapid ventricle pacing continued for an additional 4 weeks. Six of the e8 beagles treated by SMCST were still alive 4 weeks after the procedure. We evaluated SMCST's cardiotherapeutic effects by comparing beagles treated by SMCST with beagles that underwent a sham operation (control, n = 5). **Results.** Diastolic function, as well as systolic function improved significantly in the SMCST group as compared with the sham group (control vs SMCST group, median [interquartile range]: E/E', 16 [0.9] vs 11 [1.0]; $P < 0.001$; tau, 47 [6.0] vs 36 [4.4] ms: $P = 0.005$. Ejection fraction, 22 (6.0) versus 46 (7.5) %, $P < 0.001$; end-systolic elastance, 2.5 (0.4) versus 8.2 (3.5) mm Hg/ml, $P = 0.001$). Histological examination revealed that the volume of collagen I and the collagen I/III ratio in the myocardium were significantly higher in the control than that in the SMCST group (collagen I, 6.0 [0.8] vs 2.6 [1.3]; $P = 0.006$; collagen I/III ratio, 4.8 [1.7] vs 1.2 [0.4]; $P = 0.010$). **Conclusions.** The potential of SMCST to ameliorate both systolic and diastolic performance was proven. The SMCST may be an alternative therapy of conventional medical treatment in the dilated cardiomyopathy heart.

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Heart transplantation[1] and left ventricular assist device implants[2] have excellent therapeutic potential, but they are limited by donor shortage and implant durability, respectively. The need for new therapies is being met in part by translational cell therapy research,[3] which has already been used to improve cardiac function in a clinical setting. Several preclinical cell therapy studies show promise in promoting the functional recovery of the heart, especially of systolic function, which is used as the primary indicator of cell therapy efficacy.

Recent studies suggest that cell therapies can improve symptoms or exercise tolerance,[4] even in the absence of evidence of functional heart recovery, as judged, primarily, by ejection fraction (EF). Although these paradoxical results could be explained by improved diastolic function,[5] these studies have not explored diastolic function or described correlations between cell therapy–induced histological changes and changes in diastolic function. Other experimental studies have reported the improvement of diastolic and systolic function after cell therapy, although they have not addressed how cell therapies affect the distressed extracellular matrix of the damaged heart.

In this study, we hypothesized that skeletal myoblast cell-sheet transplantation (SMCST) could improve not only systolic function but also diastolic function after heart failure following the histological change on the impaired myocardium.

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T.S. performed all the experiments, analyzed all the data, and made the article and figure. S.M. contributed to make the arrangement of all the experiments and analyze the data. S.F. was one of contributors for analyzing the data about diastolic functions. N.K. was an adviser for histopathological experiments in this study. S.N. was adviser for analyzing the cardiac function of experimental animals via speckle-tracking echocardiography. T.D. was a contributor for statistical analysis of all the data in this experiment. Y.O. was a contributor for analyzing the data about diastolic functions. Y.S. is the corresponding author and helped the first author design the experiments in technical aspects.

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MATERIALS AND METHODS

All studies were approved by Osaka University's Institutional Ethics Committee. All animals were handled in accord with the Principles of Laboratory Animal Care (the National Society for Medical Research) and the Guide for the Care and Use of Laboratory Animals (an NIH publication). All procedures and analyses were blinded. The authors had full access to and take full responsibility for the integrity of the data and agree to the manuscript as written.

Preparation of Skeletal Myoblast Cell Sheets

Primary skeletal myoblasts isolated from 10-kg female beagles (Oriental Yeast Co. Ltd, Tokyo, Japan) were cultured and expanded in vitro as reported previously.[6] Briefly, 9.0 g of skeletal muscle was removed from the musculus biceps femoris, minced, and incubated with 0.5% type I collagenase (Gibco, Grand Island, NY) in Dulbecco modified Eagle medium (Gibco) for 40 minutes at 37°C. Excessive connective tissues were removed using 26G needles to minimize the contaminating fibroblasts with the addition of trypsin-EDTA (Gibco). Skeletal myoblasts were seeded into five 150-cm^2 polystyrene flasks and cultured in SkBM (Cambrex, Walkersville, MD) supplemented with 10% fetal bovine serum (ThermoTrace, Melbourne, Australia) for 10 days at 37°C. During the culture process, the cell densities were maintained at less than 70% confluence by carrying out passaging of cells for 1 time to avoid premature differentiation of skeletal myoblasts. The skeletal myoblasts were dissociated with trypsin-EDTA, placed in 60-mm temperature-responsive culture dishes (Cellseed, Tokyo, Japan) (2.0×10^6 SMBs per dish), and cultured again for 24 hours at 37°C. The dishes were then incubated at 20°C, causing the skeletal myoblasts to spontaneously detach as a scaffold-free sheet.

Generation of a Canine DCM Model, and SMCST

Twenty-two female beagles were endotracheally intubated under general anesthesia. The heart was exposed via the left fifth intercostal space and 2 bipolar pacing leads (Fineline II EZ Sterox; Boston Scientific, Boston, MA) were attached to the right ventricle and connected to a pulse generator (Insignia I; Boston Scientific). The ventricle was continuously paced at 240 beats per minute. After 4 weeks, the 18 surviving beagles were randomly divided into 2 groups: 10 beagles did not receive any treatment, and the other 8 received 20 skeletal myoblast cell sheets onto the left ventricle (LV) lateral wall. Only the LV lateral wall, designated as the "treatment side," was treated; the contralateral septal wall (anteroseptal wall) was designated as the "remote side." Rapid pacing was temporarily discontinued during the injection or transplant procedures, but resumed the following day and continued for an additional 4 weeks. At the end of this period, 5 of the beagles treated by sham operation (control) and 6 of the beagles

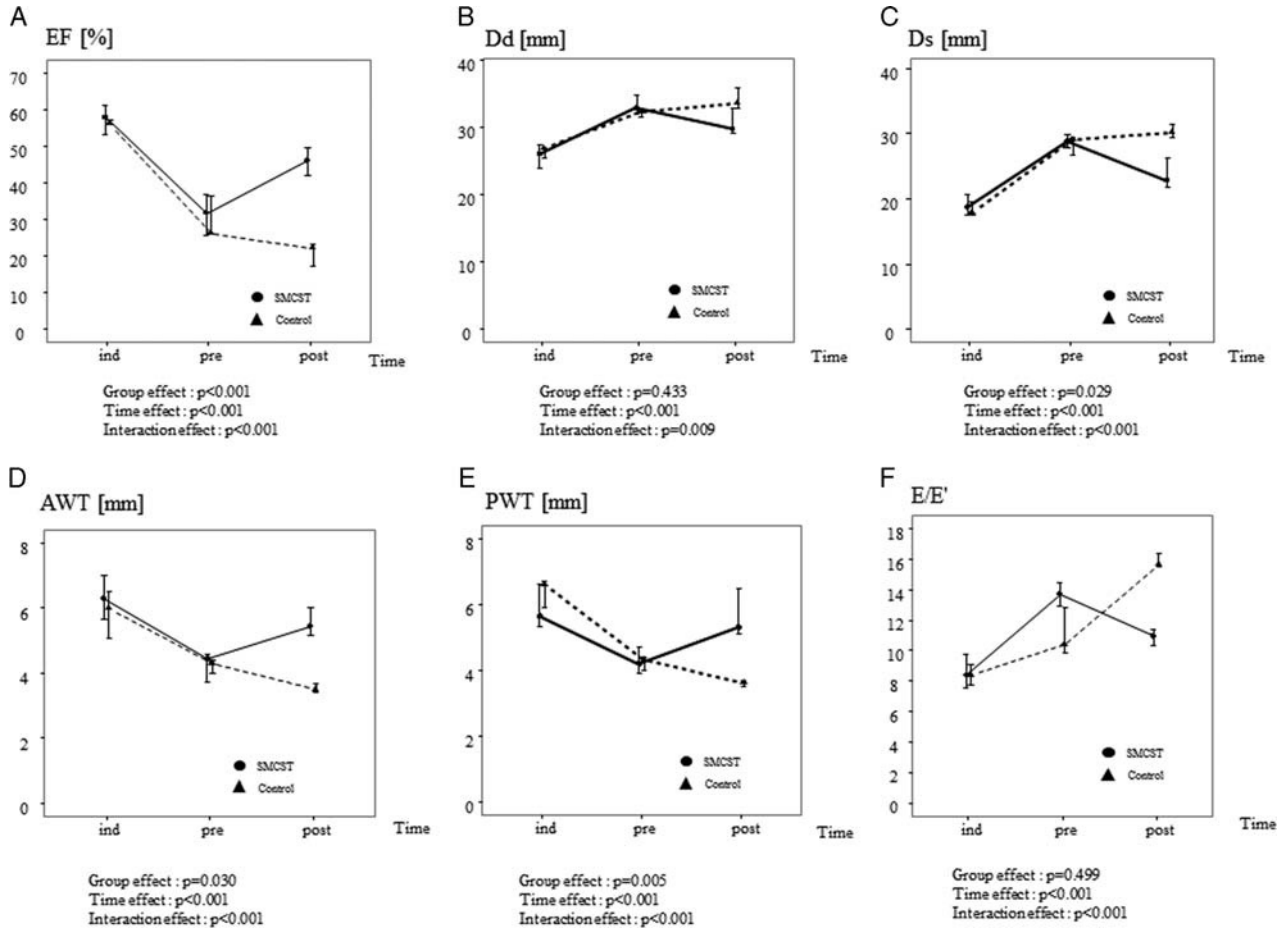

FIGURE 1. Standard echocardiography showed significant LV reverse remodeling in the SMCST group compared with control. Dd, LV end-diastolic dimension; Ds, LV end-systolic dimension; AWT, LV anterior-wall thickness; PWT, LV posterior-wall thickness; Ind, induction of rapid-pacing; Pre, PLGA or SMCST pre-treatment; Post, PLGA or SMCST posttreatment. Median with IQR is shown. IQR indicates interquartile range.

treated by SMCST were still alive. We retrieved and examined hearts from beagles in the control and SMCST groups 4 weeks after SMCST.

Standard Echocardiography, Speckle-Tracking Echocardiography, and Cardiac Catheterization

Echocardiography (Altida, Toshiba Medical Systems Corporation, Tochigi, Japan) was performed under general anesthesia. Standard echocardiography was used to measure EF, E/E′, end-diastolic and end-systolic dimensions, and LV anterior and posterior wall thickness. We used speckle-tracking echocardiography (STE) and the offline software Artida Extend (Toshiba Medical Systems Corporation) to measure radial, circumferential, transverse, and longitudinal strain, quantitatively assessing the regional LV wall motion. We also evaluated the peak early systolic and diastolic circumferential strain rates (Ssr and Esr, respectively) and calculated the regional diastolic function from the spatial derivative of the velocity in each segment.

Cardiac catheterization was performed under general anesthesia 4 weeks after SMCST or sham treatment. A 3-Fr micromanometer-tipped catheter (SPR-249; Millar Instruments, Houston) was inserted through the LV apex to measure heart rate, LV maximal systolic pressure (BP max), the maximum rate of change in LV pressure (dP/dt max), the time constant of LV relaxation (τ: tau), and the end-diastolic pressure (EDP). We also analyzed the pressure-volume loops using a 4-Fr 6-electrode conductance electrode catheter (Unique Medical Co., Tokyo, Japan) to evaluate Ees.

Histological Analysis

Hearts were sliced transversely, fixed with 10% buffered formalin, and embedded in paraffin. The sections were stained with hematoxylin-eosin, Masson trichrome, picrosirius red, and periodic acid-Schiff. The interstitial fibrotic area stained by picrosirius red and the intramyocardial fibrotic area stained by antibodies against collagen I (Abcam, Cambridge, UK) and collagen III (Novus Biologicals, Littleton, CO) were calculated using planimetry with the morphometry analyzer NIS-elements D (Nikon, Japan).

Statistical Analysis

Data are summarized as median with interquartile range. Analyses were performed with nonparametric approaches because the sample sizes were too small to allow for the checking of assumptions for parametric approaches. Comparisons between 2 groups were made using the Wilcoxon-Mann-Whitney *U* test. Serial hemodynamic and speckle-tracking echocardiographic data were compared between the control and SMCST groups by nonparametric repeated measures analysis[7] with the main effects of group and time and the interaction effect of group × time. Histopathological findings

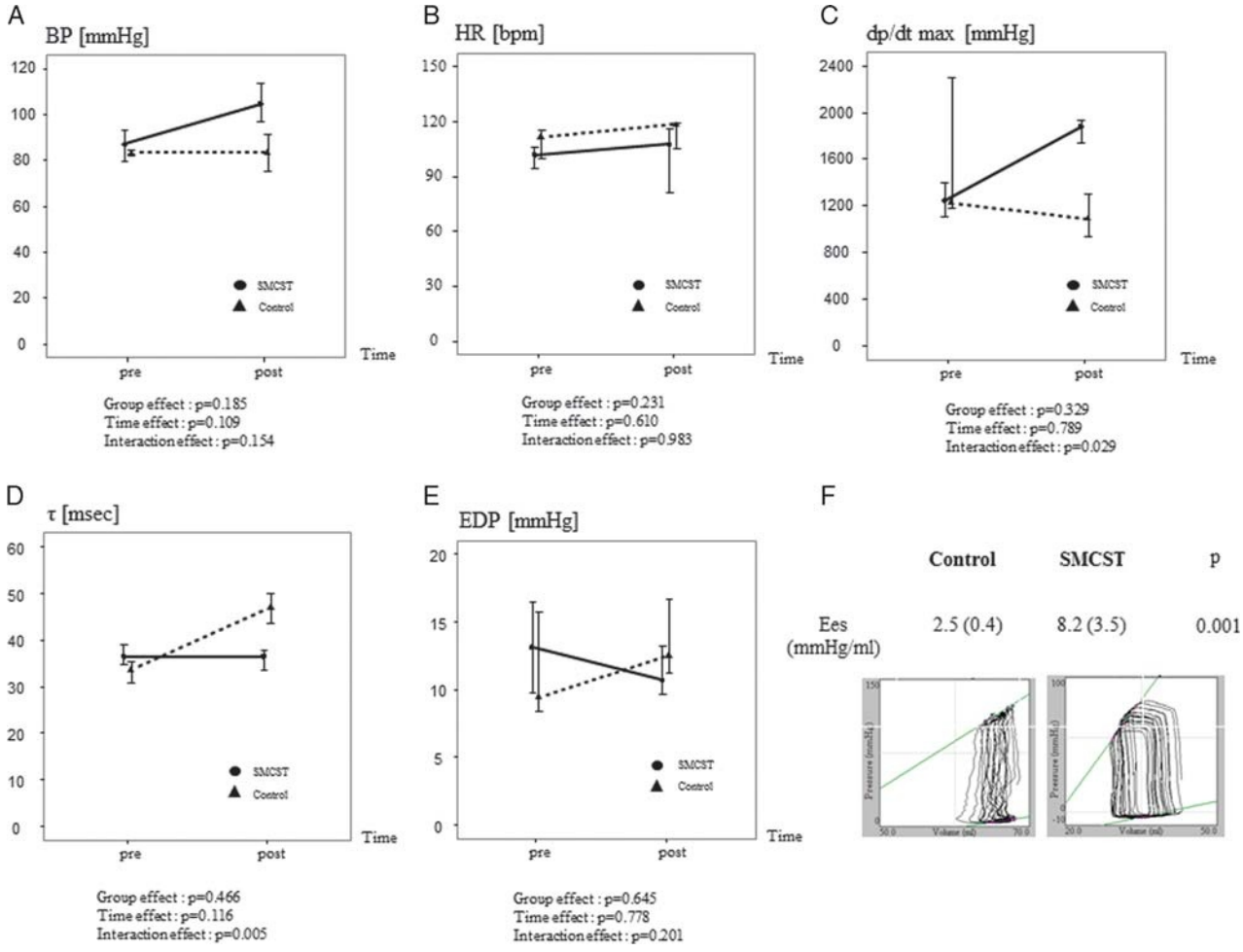

FIGURE 2. Catheterization study results. F, Note the difference in pressure-volume loops between the control and SMCST groups. dp/dt max, maximal rate of LV pressure change; τ(tau), isovolumic relaxation constant. Median with IQR is shown. IQR indicates interquartile range.

paired at the treatment and remote sides were also compared between both the groups by the nonparametric repeated measures analysis with the main effects of group and side and the interaction effect of group × side. All values are 2-sided and values of *P* less than 0.05 were considered to indicate statistical significance. Statistical analyses were performed with the R program (version 2.12.2; R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

We generated a canine dilated cardiomyopathy (DCM) heart failure model in 18 beagles by continuous rapid pacing[6] of the ventricle for 4 weeks, after which 10 beagles were treated by a sham operation (control) and 8 by SMCST. Rapid ventricle pacing continued for an additional 4 weeks. At the end of this period, we compared changes in histological features and hemodynamics between the surviving beagles in the control (n = 5) and SMCST (n = 6) groups. To evaluate the influence of SMCST on different regions of the LV, we defined the cell-sheet transplant area (the lateral wall) as the treatment side, and the region furthest from the transplant site (the anteroseptal wall) as the remote side.

SMCST Enhanced Global Functional Recovery

After 4 weeks of rapid ventricle pacing, cardiac performance had deteriorated markedly in all of the dogs. In the control group, systolic and diastolic cardiac function continued to deteriorate in the 4 weeks after the sham operation. In contrast, these functions were preserved or improved in the SMCST group: control versus SMCST EF was 22 (6.0) versus 46 (7.5) %, $P < 0.001$; E/E' was 16 (0.9) versus 11 (1.0), $P < 0.001$. The LV remodeling was reversed after SMCST but not after the sham operation: control versus SMCST LV end-diastolic volume was 34 (3.0) versus 30 (3.6) mm, $P = 0.009$; LV end-systolic volume was 30 (2.1) versus 23 (4.4) mm, $P < 0.001$; LV anterior wall thickness was 3.5 (0.3) versus 5.5 (0.8) mm, $P < 0.001$; and LV posterior wall thickness was 3.6 (0.2) versus 5.3 (1.4) mm, $P < 0.001$ (Figure 1).

Catheterization studies showed that the SMCST group had a significantly larger end-systolic elastance (Ees) and max dp/dt max than the control group, and a significantly smaller tau: control versus SMCST Ees was 2.5 (0.4) versus 8.2 (3.5) mm Hg/mL, $P = 0.001$; dp/dt max was 1080 (364) versus 1875 (196) mm Hg, $p = 0.029$; and tau was 47 (6.0) versus 36 (4.4) msec, $P = 0.005$. The EDP was smaller after SMCST than after the sham operation, although the difference was not statistically significant: control versus SMCST EDP was 13(5.5) versus 11 (3.6) mm Hg, $P = 0.201$. Maximum blood pressure (BPmax) and heart rate did not differ significantly between the groups (Figure 2).

Regional Functional Recovery After SMCST

Serial changes in regional systolic and diastolic cardiac function were assessed by strain and strain-rate, respectively,

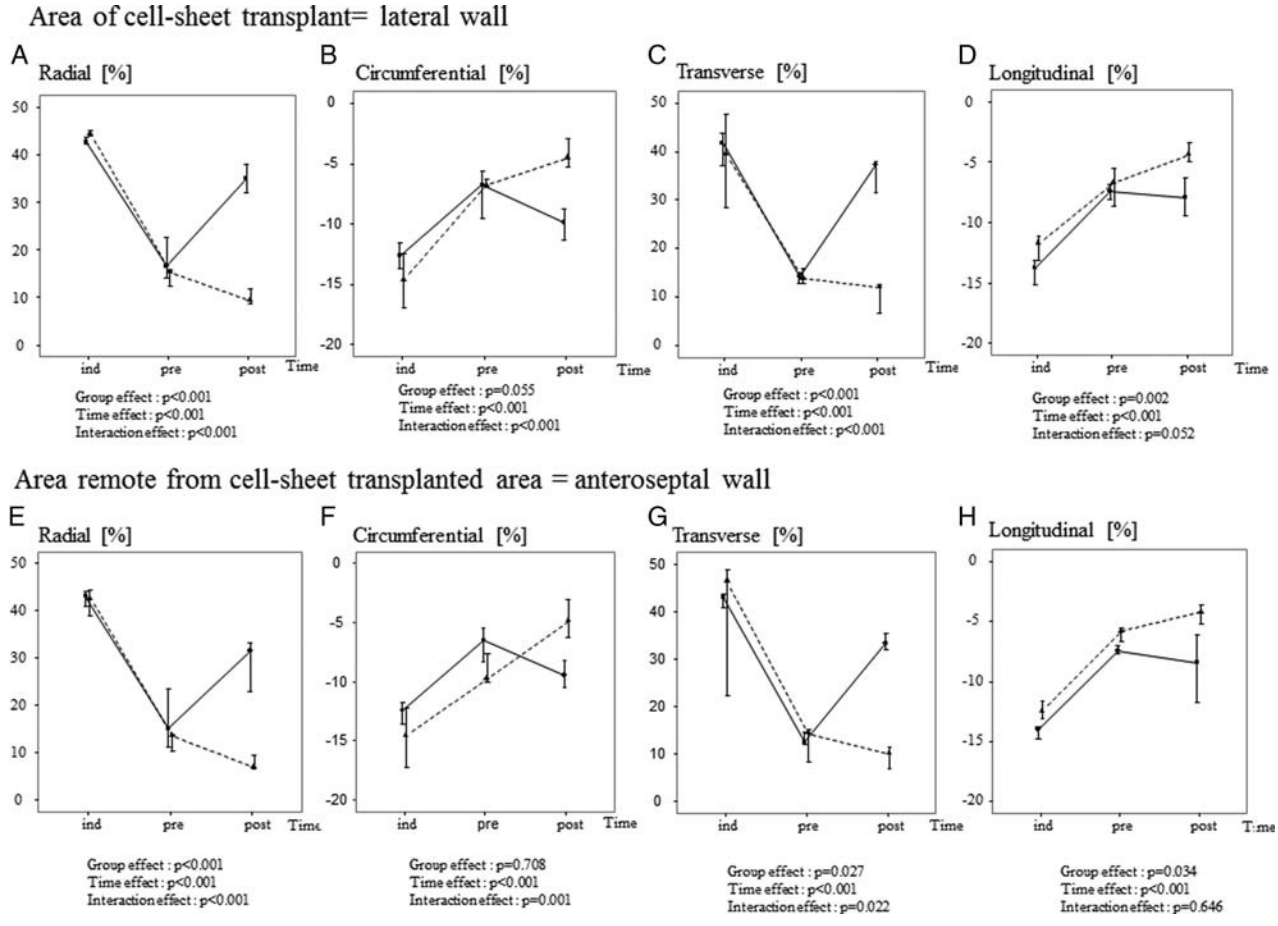

FIGURE 3. Regional analysis of myocardial systolic function. A-D, On the treatment side, all strain values were larger after SMCST than after the sham operation. E-H, Absolute values of the radial, circumferential, and transverse strains on the remote side also recovered significantly after SMCST. Median with IQR is shown. IQR indicates interquartile range.

with STE.[8,9] All strain values had decreased after the initial 4 weeks of continuous rapid pacing. Four weeks after sham treatment, further significant decreases were seen in the peak systolic radial, circumferential, transverse, and longitudinal strain values at both the treatment and remote sides. In contrast, 4 weeks after SMCST, all strain values were preserved or had improved.

Absolute values of strain measured at the treatment side (Figures 3A-D) were greater after SMCST than after the sham operation: control versus SMCST values for treatment-side myocardial strain were radial 9.3 (3.3) versus 35 (5.9) %, $P < 0.001$; circumferential, –4.5 (2.5) versus –9.8 (2.7) %, $P < 0.001$; transverse, 12 (5.9) versus 37 (6.7) %, $P < 0.001$; and longitudinal –4.3 (1.6) versus –7.9 (3.1) %, $P = 0.052$. The absolute radial, circumferential, and transverse strain values at the remote side (Figures 3E-G) recovered significantly after SMCST but not after the sham operation: control versus SMCST radial values were 6.9 (2.9) versus 32 (10) %, $P < 0.001$; circumferential, –4.9 (3.1) versus −9.5 (2.3) %, $P = 0.001$; and transverse, 10 (4.6) versus 33 (3.5), $P = 0.022$.

The absolute value of the strain rate in the Ssr improved significantly at both the treatment and remote sides after SMCST, whereas no recovery was seen after the sham operation: control versus SMCST Ssr at the treatment side was 0.6 (0.08) versus 1.0 (0.4) s^{-1}, $P = 0.008$ (Figure 4A); Ssr at the remote side was 0.7 (0.1) versus 1.0 (0.1) s^{-1}, $P = 0.004$ (Figure 4C). This trend coincided with the strain rate in the Esr in the control and SMCST groups: control versus SMCST treatment-side Esr was 0.8 (0.2) versus 1.6 (0.4) s^{-1}, $P < 0.001$ (Figure 4B); remote-side Esr was 0.8 (0.3) versus 1.5 (0.5) s^{-1}, $P = 0.002$ (Figure 4D).

Histological Findings of Reverse LV Remodeling After SMCST

We examined the gross myocardial structure 4 weeks after treatment. The hematoxylin-eosin and Masson trichrome (Figures 5A-D) showed a thicker LV wall and a smaller LV cavity after SMCST than after the sham operation. Picrosirius red staining showed significantly less interstitial fibrosis, on both the treatment and remote sides, after SMCST than after the sham operation: the percentage of fibrosis on the treatment side (lateral wall) in control versus SMCST was 25 (1.4) versus 10 (1.3) %; on the remote side (anteroseptal wall), it was 24 (2.3) versus 12 (1.2) %; interaction effect, $P = 0.011$ (Figures 5G-K).

The control group had significantly larger amounts of collagen I in the myocardium than the SMCST group (6.0 [0.8] vs 2.6 [1.3], $P = 0.006$; Figure 6A), and significantly less collagen III (1.2 [0.1] vs 2.5 [0.4], $P = 0.006$; Figure 6B). The ratio of collagen I to collagen III was significantly larger in the control than in the SMCST group (collagen I/III ratio, 4.8 [1.7] vs 1.2 [0.4], $P = 0.010$). The collagen I/III ratio significantly correlated

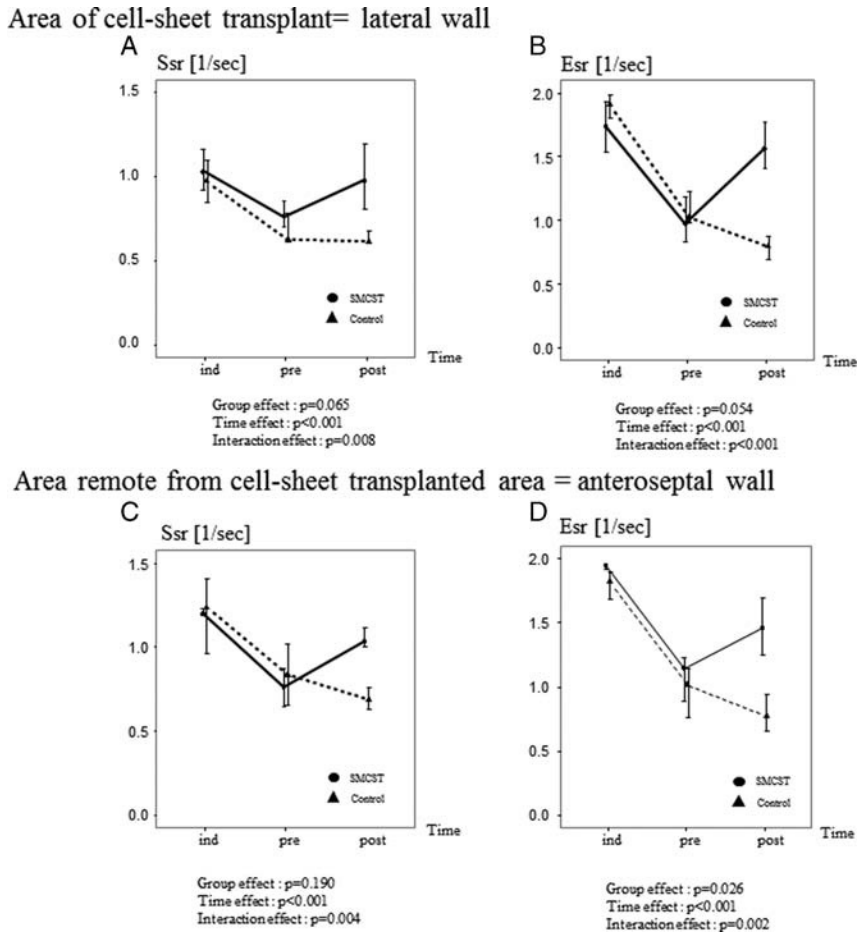

FIGURE 4. Regional diastolic function of the remote side recovered to almost that of the treatment side in the SMCST group, and both sides showed significantly better recovery than the corresponding areas in the control group (A-D). Median with IQR is shown. IQR indicates interquartile range.

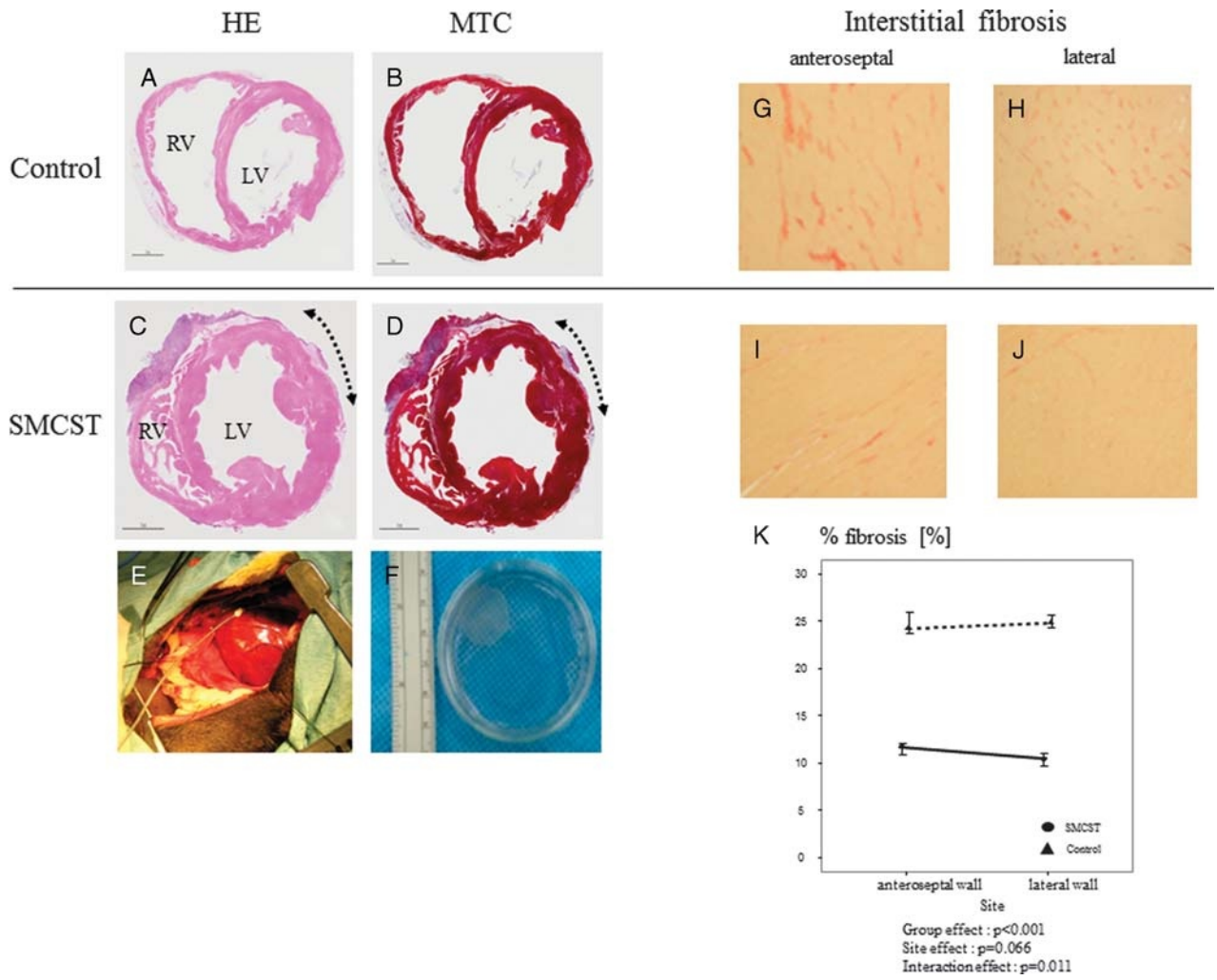

FIGURE 5. Representative micrographs show H&E and MTC staining for (A, B) the control group and (C, D) the SMCST group (arrow indicates the cell sheet transplant area). E, Photos of the surgery to induce rapid pacing. F, The canine skeletal myoblast cell sheet. G-J, A representative micrograph of interstitial fibrosis in control (G, H) and SMCST (I, J) tissues; (K) fibrosis was significantly less after SMCST than after the sham surgery. H&E indicates hematoxylin-eosin, MTC, Masson trichrome.

with EDP, and Ssr as a whole although that in each group did not (EDP: overall: $\rho = 0.78$, $P = 0.005$; control, $\rho = 0.927$, $P = 0.037$. SMCST: $\rho = 0.384$, $P = 0.397$; Figure 6C). Ssr: overall: $\rho = 0.84$, $P < 0.0001$. Control: $\rho = 0.881$, $P = 0.037$. SMCST: $\rho = 0.662$, $P = 0.329$ (Figure 6D).

DISCUSSION

In this study, standard echocardiography and catheterization analysis showed that SMCST significantly improved global systolic and diastolic functions as compared with a sham operation. The STE demonstrated the recovery of regional systolic and diastolic function in the SMCST group, as shown by the change of strain and strain rate. These functional gains might result from histological changes, such as the regulation of interstitial fibrosis. We hypothesized that the simultaneous recovery of systolic contraction but relaxation during diastolic phase would be a key to significant improvement of impaired myocardium because each of them is originally a complement. The STE[8,9] was very suitable for clearing this.

The STE[8,9] is a new device for evaluating more accurate data of regional myocardial dynamics different from a conventional echocardiography, and the accuracy and the validity of this approach have been already established. Before, the extension of therapeutic effect of cell sheet had been thought to be very limited to the area that was attached. However, with this new approach, it was proven that the healing effect of SMCST was much more extended than we had expected, almost the whole area of impaired myocardium of LV.

Diastolic dysfunction is caused by the deterioration of myocardial relaxation and by myocardial stiffness.[10] It has been recently reported that alleviation of symptom of congestive heart failure is more related to the improvement of diastolic function than that of the systolic function.[4] It is also important to recognize that target of SMCST are not only systolic function but also diastolic function of LV because clinically, LV systolic dysfunction is thought to be accompanied by LV diastolic dysfunction.[11] In this respect, the recovery of diastolic function by SMCST that has never been paid attention to in the field of cell therapy seems valuable and clinically significant. The essence of healing effect of SMCST may not be only for the systolic functional recovery but also diastolic one.

The choice of delivery route of the cell is also quite important. Cell implantation by needle injection has lower potential

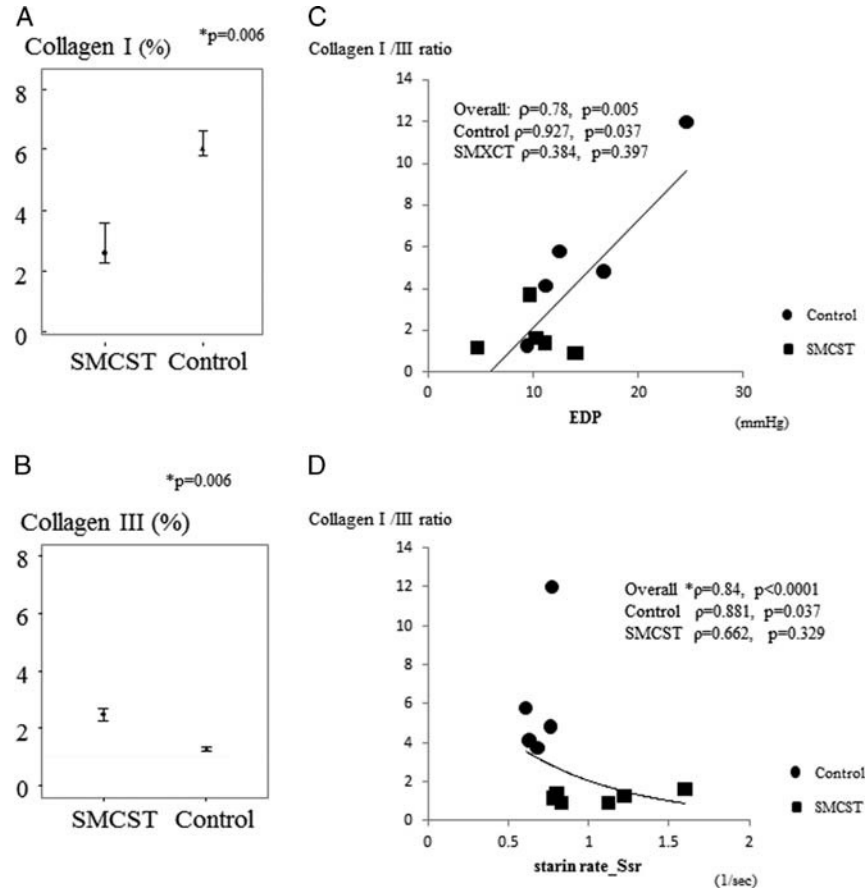

FIGURE 6. Note the correlation between the collagen I/III ratio and diastolic parameter. Myocardium from the control group contained significantly more collagen I than myocardium from the SMCST group (A, control vs SMCST, collagen I was 6.0 (0.8) vs 2.6 (1.3), $P = 0.006$), whereas collagen III was significantly lower in the control than in the SMCST group (B, control vs SMCST, collagen III was 1.2 [0.1] vs 2.5 [0.4]; $P = 0.006$). The collagen I/III ratio significantly correlated with EDP and Ssr although those in each group did not (EDP: overall: $\rho = 0.78$, $P = 0.005$; Control: $\rho = 0.927$, $P = 0.037$; SMCST: $\rho = 0.384$, $P = 0.397$; Ssr: overall: $\rho = 0.84$, $P < 0.0001$; control: $\rho = 0.881$, $P = 0.037$; SMCST: $\rho = 0.662$, $P = 0.329$; C, D, respectively).

of inducing myocardial regeneration whereas sheet implantation has been proven to be more advantageous in the recruitment of endogenous cytokines or stem cell recruitment and lower potential of arrhythmia.[12]

One of the most important points in the present study is SMCST which showed a cardioprotective influence on the impaired myocardium not regionally but more widely. It has been reported through our previous studies[4,6] that intramyocardial expression of a variety of mRNA of endogenous cytokines, such as hepatocyte growth factor and vascular endothelial growth factor after SMCST, yield antifibrotic effect and angiogenesis, and this phenomenon potentially relate to restoration of impaired myocardium. The extension of these healing effects is thought to be so large as to cover whole LV myocardium.[12] These cytokine secreted from transplanted cell sheet is expected to permeate into the myocardium and the blood stream via capillary, and finally influence the global function of LV.

Another important point in the current study is that controlling collagen deposits and the ratio of collagen I to collage III may be crucial for resolving LV dysfunction. The importance of the collagen type I/III ratio in LV remodeling has been demonstrated in experimental models,[13,14] and a role for the regulation of collagen I, which provides tensile strength and stiffness,[15] in LV function has been proposed.[16] However, it has been unclear how collagen III, which provides storage and release of elastic energy, contributes to the hemodynamic changes that accompany the repair of the damaged myocardium.

Collagen III, different from tougher collagen I, is composed of reticular fiber. Progression of myocardial stiffness is brought by the imbalance of the volume of collagen I and collagen III, and this phenomenon is brought by the replacement of collagen III with collagen I. However, clarifying the mechanisms of collagen turnover is made more difficult by the changes seen in the collagen I/III ratio as the myocardium heals, as demonstrated in an induced hypertension model.[13,14] In hypertensive rats, the collagen I/III ratio decreases 10 weeks after the start of the experiment, but it shows an increase at 84 weeks, because of the transition from compensated to decompensated LV hypertrophy.[13] In our opinion, changes in the volume of collagen I, a primary component of myocardial extracellular matrix,[17] strongly affect myocardial stiffness.

In conclusion, therapeutic target of heart failure may be both systolic and diastolic function, and cell therapy also should target distressed myocardium demonstrating on both of them. The SMCST has the potential to improve both systolic and diastolic function in the severely damaged heart, especially by controlling the collagen I/III balance. Our findings

indicate that SMCST may be an alternative therapy of conventional medical treatment in the DCM heart even in clinical situations.

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