

Novel Injectable Bioartificial Tissue Facilitates Targeted, Less Invasive, Large-Scale Tissue Restoration on the Beating Heart After Myocardial Injury

Theo Kofidis, MD; Darren R. Lebl, MS; Eliana C. Martinez, MD; Grant Hoyt, RA; Masashi Tanaka, MD; Robert C. Robbins, MD

Background—Implantation of bioartificial patches distorts myocardial geometry, and functional improvement of the recipient heart is usually attributed to reactive angiogenesis around the graft. With the liquid bioartificial tissue compound used in this study, we achieved targeted large-scale support of the infarcted left ventricular wall and improvement of heart function.

Methods and Results—A liquid compound consisting of growth factor-free Matrigel and 10^6 green fluorescent protein (GFP)-positive mouse (129sv) embryonic stem cells (ESCs) was generated and injected into the area of ischemia after ligation of the left anterior descending artery in BALB/c mice (group I). Left anterior descending artery-ligated mice (group II) and mice with Matrigel (group III) or ESC treatment alone (group IV) were used as the control groups ($n=5$ in all groups). The hearts were harvested for histology 2 weeks later after echocardiographic assessment with a 15-MHz probe. The liquid injectable tissue solidified at body temperature and retained the geometry of the infarcted lateral wall. Immunofluorescence stains revealed voluminous GFP grafts. The quality of restoration (graft/infarct area ratio) was $45.5 \pm 10.8\%$ in group I and $29.1 \pm 6.7\%$ in group IV ($P=0.034$). ESCs expressed connexin 43 at intercellular contact sites. The mice treated with the compound had a superior heart function compared with the controls ($P<0.0001$ by ANOVA/Bonferroni test; group I: 27.1 ± 5.4 , group II: 11.9 ± 2.4 , group III: 16.2 ± 2.8 , group IV: 19.1 ± 2.7).

Conclusions—Injectable bioartificial tissue restores the heart's geometry and function in a targeted and nondistorting fashion. This new method paves the way for novel interventional approaches to myocardial repair, using both stem cells and matrices. (*Circulation*. 2005;112[suppl I]:I-173–I-177.)

Key Words: heart failure ■ myocardium ■ cells ■ transplantation

There is an increasing body of experimental approaches to restore/regenerate failing myocardium. Two of the most promising pathways are direct implantation of primordial types of cells into the injured heart and the replacement of portions of the heart muscle with tissue-engineered bioartificial grafts. Both techniques display advantages and limitations. The direct transfer of pluripotent stem cells into the heart has been reported to improve cardiac function of the recipient animals after myocardial injury.^{1–3} Limitations are arrhythmogenicity, immunogenicity, and tumorigenicity of the injected cells, as well as their insufficient potential to survive, engraft, and differentiate to the cardiac cell phenotypes.^{4,5}

Replacement of heart muscle—the tissue engineering approach—is also associated with a series of limitations. The engineered tissue needs to be robust enough to withstand high amounts of circumferential wall stress during the cardiac cycle.⁶ Moreover, the heart constitutes a complex helical structure^{6,7} with significant local asymmetry and anisotropy.

The contractions of the particular elements of all portions of the ventricle have to be in unison and perfect coordination to guarantee maximal possible output. The majority of the previously developed bioartificial matrices lack a heart-like microstructure. They are devoid of a vascular tree or microvasculature and are destined to die after implantation into the ischemic heart. The heart's functional improvement after cell or tissue transfer is usually attributed to secondary angiogenesis because of the lack of a convincing explanation of the mechanism of engraftment and participation in contractile activity.⁸ Ideally, a bioartificial tissue should be pliable enough to allow for a homogeneous mixture with stem cells, engraft in a fashion that would not distort the geometry of the left ventricle, and retain its wall thickness, thereby preventing aneurysm formation.

Keeping that in mind, it becomes clear that surgical research will have to seek novel techniques to manufacture portions of the heart and later an entire heart chamber. An adaptable, potentially resorbable carrier of viable matter

From Cardiothoracic Surgery (T.K., D.R.L., G.H., M.T., R.C.R.) and Pediatric Cardiology (E.C.M.), Stanford University Medical School, Stanford, Calif.

Correspondence and reprint requests to Theo Kofidis, MD, Thoracic and Cardiovascular Surgery, Hannover Medical School, Carl Neuberg Str. 1, 30625 Hannover, Germany. E-mail Kofidis@thg.mh-hannover.de

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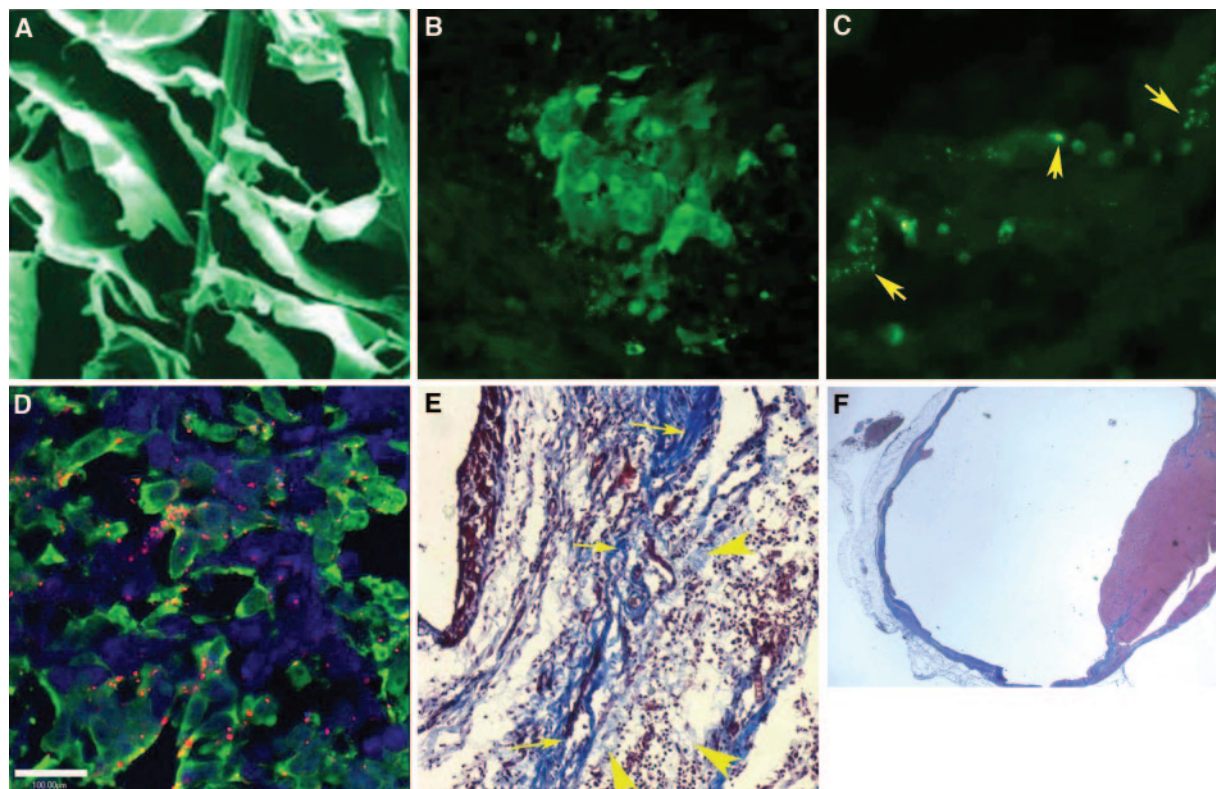


Figure 1. A, Matrigel is a porous liquid collagen compound that solidifies to a gel consistency few hours after injection into an environment of 37°C. B, The injected liquid tissue forms GFP-positive conglomerates containing dense donor cell colonies within the area of injury (immunofluorescence microscope, $\times 50$). C, Scar myocardium serves as a control, observed with the same filter as in B. The arrows point at exceedingly bright spots that represent autofluorescence. D, The cells express connexin 43 (red spots, as stained by Texas Red, indicating the presence of gap junctions) at what appears to be connection sites to neighboring donor and host cells, hence indicating eventual communication between these cells (confocal colocalization studies, $\times 650$). E, Trichrome staining shows a cross-section of the injured left ventricular wall with Matrigel remains (a significant portion is being washed out at the fixation process) and resident cells. F, Control mouse hearts without treatment, showing extensive scars in the area of injury, as well as extensive aneurysm formation (trichrome stain).

(primordial cells) needs to be developed that will engraft after implantation without distorting myocardial geometry and adversely affecting hemodynamics. A robust cell line has to be introduced that will have the potential to survive, engraft, undergo functional junctions with the injured vicinity of the host heart, and improve its diminished function.

In the present work we hypothesize that liquid bioartificial tissue will facilitate large-scale myocardial restoration of the beating heart and improve cardiac function without distortion of the left ventricular architecture in a model of acute myocardial damage. Furthermore, we wish to miniaturize tissue engineering-based myocardial restoration to the scale of the mouse heart without death due to severe bleeding.

Methods

Animal Care

All surgical interventions and animal care were provided in accordance with the Laboratory Animal Welfare Act, the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, publication number 78-23, revised 1978), and the *Guidelines and Policies for the Use of Laboratory Animals for Research and Teaching of the Department of Comparative Medicine*, Stanford University School of Medicine. The animals were euthanized after functional measurements in deep inhalational anesthesia with isoflurane, in compliance with the above guidelines.

Compound Preparation and Labeling

Human elongation factor 1 α -Promoter (pEF1 α)-enhanced green fluorescent protein (EGFP), which contains EGFP gene under the control of human EF1 α promoter and a neomycin resistance cassette, was constructed as follows: The promoter region of pEGFP-N3 (Clontech) was removed by removing the Ase I-Nhe I DNA fragment and joining the blunt-ended termini human EF1 α promoter from pEF-BOS site of the plasmid. D3 endothelial stem cells were transfected with pEF-1 α -EGFP and one clone, brightly expressing EGFP, was chosen and used for the experiments. The clone was adapted to feeder-free conditions and mixed with liquid growth factor reduced Matrigel (BD Bioscience). Matrigel has the physical property of consolidating to a gel consistency at 37°C in a few hours (Figure 1A). The mixture was injected intramyocardially into the area of acute ischemia after ligation of the left anterior descending artery (LAD).

Animal Groups

In group I (n=5), BALB/c mice received 50 μ L of the mixture into the area of myocardial injury. Group II mice underwent LAD ligation only (n=5). In group III, Matrigel alone was injected into the area of myocardial injury (n=5). In group IV, only endothelial stem cells were injected (n=5).

Animal Anesthesia

Mice were pre-anesthetized with isoflurane and received an intraperitoneal injection of ketanest/xylazine (50 mg/kg). The animals were then intubated and ventilated for the entire length of the procedure. The surgical approach involved a left lateral thoracotomy,

pericardectomy (the mouse pericardium is merely a transparent membrane, and removing it or incising it is inevitable if a cardiac procedure is intended), and identification of the LAD for ligation. Once the pericardium is opened in a mouse, the left atrium can be seen contracting vigorously. From a lateral approach, one looks for the middle of the free margin of the left atrium. This is the point at which the surgeon usually identifies the LAD and moves distally to the transition from the first to the second third of the vessel course on the surface of the LAD. This can be viewed as the optimal spot for the LAD ligation to obtain a significant infarction of the mentioned magnitude. Ligation in the immediate proximity of the left atrial margin (too proximal) usually causes death of the animal. A ligation further distally will cause a much too small infarction that will not impact left ventricular function sufficiently. After ligation of the LAD, 10^6 donor cells in 25 μL medium were mixed with 25 μL of liquid Matrigel.

Injections

The resulting total compound volume was 50 μL . The injection was targeted at the area of injury that bleaches out immediately after ligation of the LAD in mice. Our experience has shown that the mouse left ventricle experiences an infarction of the magnitude of 40% to 50% of the left ventricular wall by this approach. The transplantation of the liquid bioartificial tissue was then performed on the beating heart of the mouse (during inhalational anesthesia, the heart rate of a BALB/c mouse ranged between 300 and 400 bpm). During targeted injection, the affected area swells slightly, an indication that the compound does not escape into the left ventricular cavity (Figure 1B).

Echocardiography

Mice were transferred in a portable anesthesia chamber and kept under inhalational isoflurane anesthesia for the duration of the echocardiogram, which took place immediately before euthanization. The Acuson Sequoia C256 echocardiography system (Acuson) with a 15.8-MHz probe was used. The following measurements were obtained: End-systolic diameter (ESD) and end-diastolic (EDD) diameter in a cross section, ESD and EDD at 2 different sites of a longitudinal section of the heart (basal at the submitral level and apical), posterior and septal wall thickness. Fractional shortening (FS) was then calculated as $\text{FS} = (\text{EDD} - \text{ESD}) / \text{EDD}$.

Histology and Immunohistochemistry

The myocardium was sectioned at 5 different transversal levels at the site of tissue necrosis, encompassing the entire lesion. On every section the infarcted area, which can be distinguished easily both with trichrome stain and the immunofluorescence stains. Dead myocardium appears dark, without nuclei (colocalization with 4,6-diamidino-2-phenylindole) as opposed to intact myocardium, which is rich in nuclei and in which cell shapes are clearly visible in the green filter. Using the mentioned program, we morphometrically measured the entire area of infarction in μm^2 . In the mice that received cells, we measured the green fluorescent area, which corresponded to the dense cellular colonies seen. We calculated a ratio between the green (graft) area and the infarct area, which we call the graft/infarct area ratio and expressed this as a percentage. We repeated these measurements 5 times for each animal and calculated a mean.

Five- μm cryosections were stained with hematoxylin and eosin or Masson's trichrome or used for immunohistochemistry. Immunostaining was performed as previously described.⁹ The antibodies used were rabbit anti-connexin 43 (Sigma), goat anti-GFP antibody (Rockland, Inc.), and rabbit anti-GFP Alexa-488 conjugated antibody (Molecular Probes). Stained tissue was examined with a Leica DMRB fluorescent microscope and a Zeiss LSM 510 two-photon confocal laser scanning microscope.

Morphometry

For all morphometric evaluations, the focused microscopic field was photographed by an adapted camera (Diagnostic Instruments Inc).

The total GFP-positive area was measured and related to the infarction area at low magnification (ratio in %). To quantify the degree of expression of specific markers, 5 random sections of the GFP-positive graft were photographed and evaluated using the Spot advanced software, version 3.4.2 (Diagnostic Instruments Inc).

Statistics

All results are expressed as mean $\pm$ SD. Data were compared and inter-group differences were analyzed by 1-way ANOVA (Analysis of Variance) with post hoc Bonferroni test, and variance ratio (F) was calculated. Statistical analyses were performed with StatView 5.0 (SAS Institute), and significance was accepted at $P < 0.05$.

Results

Engraftment and Myocardial Restoration

Voluminous injection into the area of injury of a beating heart was feasible in the mouse model. No embolizations, loss of compound matter in the circulation, or distortion of the left ventricular structure occurred. No intraoperative deaths occurred. The injections were feasible without great effort of the beating heart of a mouse. The overall postoperative mortality was consistently 25%. We therefore operated on more animals than necessary for the completion of 1 animal group to ensure we had the appropriate number of survivors until the end of the follow-up period. Mortality was comparable in all groups. Cell injection did not affect mortality during the observation period of 4 weeks.

After injection, the liquid compound engrafted homogeneously within the injured heart muscle and adapted to the host organ collagen structure without distorting cardiac wall geometry. Confocal microscopy revealed voluminous GFP grafts. The cells embedded in liquid Matrigel formed GFP-positive colonies within the infarcted area (Figure 1B and 1C), and connexin 43 expression occurred at various intercellular contact sites to neighboring cells of both donor and host (Figure 1D). Signs of cellular atypia, nuclear polymorphism, or teratoma formation were not observed in this group. In the control group with LAD ligation and no treatment, an extensive scar formed after 2 weeks (Figure 1F).

The restorative effect (Figure 2A) ranged between 23% to 55% of the injured area, with a graft/infarct area ratio mean of $45.5\% \pm 10.8\%$ in the mice that received transplantation of the liquid compound (group I) and $29.1\% \pm 6.7\%$ in those treated with cells only (group IV; $P = 0.034$).

Cardiac Function

Echocardiography revealed superior heart function in the mice that were treated with the liquid compound compared with the controls (variance ratio [F]): 16.40, $P < 0.0001$, 1-way ANOVA). The hearts' FS in the treated and control groups was as follows: Group I, 27.1 ± 5.4 ; group II, 11.9 ± 2.4 ; group III, 16.2 ± 2.8 ; and group IV, 19.1 ± 2.7 (Figure 2B). The group treated with cells only (group IV) also showed a significantly higher FS compared with the control group with LAD-ligation and the control group that received only Matrigel ($P < 0.05$, Bonferroni post-hoc test). Lateral wall (the site of injection) thickness was 0.8 ± 0.05 mm in group I, 0.5 ± 0.06 mm in group II, 0.7 ± 0.1 mm in group III, and 0.7 ± 0.06 mm in group IV. The lateral wall was the thinnest in the infarcted group of mice that did not receive any

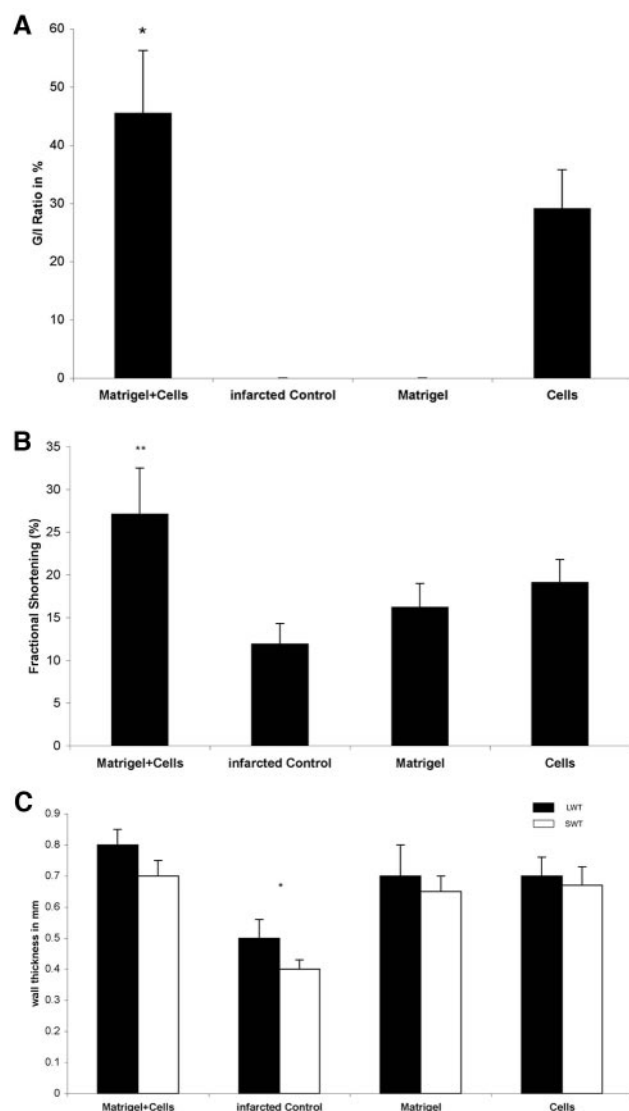


Figure 2. A, Myocardial restoration is superior in the group that received the compound of cells and Matrigel compared with those that received cells alone. Graft/infarct ratio is highest in the group treated with injectable Matrigel and cells ($P=0.034$). Of note, the graft/infarct ratio cannot be evaluated in the groups that received Matrigel only or those which were infarcted without further treatment, as in these 2 groups the green conglomerates are missing (no donor cell injection). B, Echocardiography shows superior fractional shortening in group I compared with the other groups 2 weeks after myocardial injury and treatment ($F: 16.40$, $P<0.0001$), indicating superior efficacy of the restorative injection when matrix and cells are injected simultaneously. C, Left ventricular wall thickness is retained in the group treated with the liquid compound and/or matrix alone. This indicates the importance of matrix support during myocardial remodeling and scar formation. Aneurysm formation was not observed in this group. The lateral wall was the thinnest in the infarcted control group ($F: 22.49$; $P<0.05$).

further treatment ($P<0.05$, $F: 22.49$). Similarly, septal wall thickness was 0.7 ± 0.05 mm in group I, 0.4 ± 0.03 in group II, 0.65 ± 0.05 in group III, and 0.67 ± 0.06 in group IV. Again, septal wall was thinnest in the infarcted group of animals that did not receive any further treatment ($P<0.5$, $F: 19.56$; Figure 2C).

Discussion

The 2 main novel observations in the present work are that liquid bioartificial myocardial tissue was injected in a targeted fashion for myocardial restoration, resulting in functional improvement, and that large scale restoration on the beating heart, without the need for extracorporeal circulation, has hereby been introduced. Moreover, the present model facilitated miniaturization of in vivo myocardial tissue engineering experimentation in the mouse. The mouse model may broaden the spectrum of experimental applications because of the abundance of transgenic and knock-out models, as well as the availability of agents for more profound experimental studies. Thus far, larger animals were required for studies on cardiac tissue engineering, a fact that is associated with higher costs and far more extensive time requirements.

The success and efficacy of myocardial tissue engineering experiments stands and falls with the viability of inoculated cells and the physical properties of the scaffolding matrix. A vast variety of 3-dimensional compounds have been introduced. Rigid matrices are hostile to seeded cells because of poor penetration of the vasculature and limited pliability, which would allow for synchronization of the compound contractile function with that of the host heart. Furthermore, clearly defined quadrangular or circular patches face limited engraftment that lies in the proximity of the host heart muscle.^{10–12} Any rigid or solid matrix interrupts the continuity of the myocardial architecture, signal transfer, and vascularization. A liquid matrix can be viewed as a revolutionary principle of organ repair because of the fact that it adapts to its environment structurally, regardless of the extent of damage.

It is likely that a rigid 3-dimensional construct constitutes an overload to the shear stress if these sheets are in the area of hibernating myocardium, because these layers are disrupted in their continuity by implanting a homogenous and free-standing patch. Liquid bioartificial tissue, however, does not damage this structure, because it integrates within the same. In parallel in vitro studies using bioluminescence imaging in mice, we found an 8-fold higher viability of the same cells in the same 96-well plate when the cells were embedded in our liquid matrix (data not shown). Of note, Matrigel alone in the present study was sufficient to retain left ventricular thickness 2 weeks after injury and to prevent further deterioration of cardiac function. This indicates that it is not exclusively the stem cells that restore myocardial function; thus, at least partially, the mechanism of functional improvement of a restored heart muscle may be the structural enhancement of the damaged area, and not necessarily the differentiation or integration potential of any given stem cells. To clarify this, further studies with various control groups using myofibrillar cells (myocytes) and “neutral cells” (fibroblasts) are of paramount importance. The latter aspect is often neglected in the field of myocardial repair by means of stem cell transfer.

A limitation of our study is that we did not measure cell populations in vivo precisely, as a cell-by-cell count in such dense conglomerates is impossible. We defined the graft/infarct ratio in the present form. There are not more accurate methods to measure the restorative effect of stem cell or

tissue transfer geometrically/morphologically. Our method of morphometrical evaluation of myocardial restoration however, is reliable and correlates to echocardiography data consistently. A further point of controversy might be the fact that we did not use Millar catheters to obtain more precise hemodynamic data. The measurements were sensitive enough to distinguish treated from untreated animals.

Both cardiology and cardiac surgery seek minimally invasive interventional methods of treatment that will reduce trauma to patients. Our approach to restore injured myocardium is the only one that combines the potential of stem cells with the structural properties of bioartificial tissue and can be applied on the beating heart by both disciplines, interventionally or thoracoscopically. This new method opens a whole new chapter in myocardial restoration, even though a great amount of work as to the exact location and time point of intramyocardial administration still has to be performed. Finally, the miniaturization of myocardial tissue engineering to the scale of the mouse by direct transfer of large amounts of tissue without massive bleeding can prove of utmost importance for the study of mechanisms of engraftment of stem cells and cell-matrix interaction. A plethora of stem cells becomes available for the restoration of various organs, research materials and kits find use, and novel bioimaging techniques (magnetic resonance imaging, micro-single photon emission tomography, positron emission tomography-computed tomography fusion, bioluminescence) become applicable.

Despite the beneficial structural and operational advantages of our approach, it still constitutes a random administration into the heart, similar to the direct stem cells transfer. The best possible way to restore injured heart muscle according to the most current state of knowledge and with the highest possible fidelity to the natural architecture of the heart, however, is to use the given host geometry and structure and adapt matrix and cell administration to the existing framework. Liquid myocardial tissue thereby facili-

tates viability support (by the inoculated cells) and prevention or attenuation of adverse remodeling (by scaffolding the damaged host structure) and may minimize the surgical trauma and treatment costs for the patient because of the beating heart/endoscopic applicability.

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