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PRE-CLINICAL RESEARCH

Minimally-Invasive Implantation of Living Tissue Engineered Heart Valves

A Comprehensive Approach From Autologous Vascular Cells to Stem Cells

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Objectives The aim of this study was to demonstrate the feasibility of combining the novel heart valve replacement technologies of: 1) tissue engineering; and 2) minimally-invasive implantation based on autologous cells and composite self-expandable biodegradable biomaterials.

Background Minimally-invasive valve replacement procedures are rapidly evolving as alternative treatment option for patients with valvular heart disease. However, currently used valve substitutes are bioprosthetic and as such have limited durability. To overcome this limitation, tissue engineering technologies provide living autologous valve replacements with regeneration and growth potential.

Methods Trileaflet heart valves fabricated from biodegradable synthetic scaffolds, integrated in self-expanding stents and seeded with autologous vascular or stem cells (bone marrow and peripheral blood), were generated in vitro using dynamic bioreactors. Subsequently, the tissue engineered heart valves (TEHV) were minimally-invasively implanted as pulmonary valve replacements in sheep. In vivo functionality was assessed by echocardiography and angiography up to 8 weeks. The tissue composition of explanted TEHV and corresponding control valves was analyzed.

Results The transapical implantations were successful in all animals. The TEHV demonstrated in vivo functionality with mobile but thickened leaflets. Histology revealed layered neotissues with endothelialized surfaces. Quantitative extracellular matrix analysis at 8 weeks showed higher values for deoxyribonucleic acid, collagen, and glycosaminoglycans compared to native valves. Mechanical profiles demonstrated sufficient tissue strength, but less pliability independent of the cell source.

Conclusions This study demonstrates the principal feasibility of merging tissue engineering and minimally-invasive valve replacement technologies. Using adult stem cells is successful, enabling minimally-invasive cell harvest. Thus, this new technology may enable a valid alternative to current bioprosthetic devices. (J Am Coll Cardiol 2010;56:510–20) © 2010 by the American College of Cardiology Foundation

Minimally-invasive valve implantation techniques are rapidly evolving as alternative treatment option for patients with valvular heart disease. These techniques are expected to have a major impact on the management of patients with valvular heart disease over the next several years (1). Various transvascular, catheter-based implantation approaches have been developed and successfully used in both experimental and clinical settings (2). Alternative minimally-invasive surgical

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techniques, such as the transapical approach, have been described as safe and successful procedures particularly for patients with more advanced atherosclerosis and/or complex heart valve pathologies (2,3). However, the currently available valve substitutes for minimally-invasive replacement procedures are bioprosthetic and as such will be associated with calcification and progressive dysfunctional degeneration similar to that of conventional nonviable tissue valves (glutaraldehyde fixed, and so forth). That suggests their primary clinical application in elderly patients (2).

Autologous, viable valve substitutes with regeneration potential would overcome the limitations of today's valve prostheses and enable the application of minimally-invasive treatment modalities also in younger patients (4,5). In recent years, tissue engineering techniques have been developed on the basis of decellularized allografts or rapidly degrading synthetic scaffold materials and autologous cells, aiming at living heart valve replacements with regeneration potential. Feasibility and functionality of such tissue engineered heart valves (TEHVs) implanted by conventional surgical procedures have been demonstrated by in vitro as well as in vivo experiments and initial clinical trials (6–12). Therefore, TEHV implanted by a minimally-invasive technique could become a viable autologous alternative to bioprosthetic valve replacements. A clinically relevant heart valve tissue engineering concept would ideally comprise both minimally-invasive techniques for cell harvest and valve implantation. Here, the first in vitro and in vivo experiences in an animal model toward a "complete" minimally-invasive heart valve tissue engineering approach based on rapidly degrading composite scaffolds and autologous vascular and adult stem cells are presented.

Abbreviations and Acronyms

DNA = deoxyribonucleic acid
eNOS = endothelial nitric oxide synthase
GAG = glycosaminoglycans
OD = outer diameter
SMA = smooth muscle actin
TEHV = tissue engineered heart valve
UTS = ultimate tensile strength

Methods

This comprehensive study comprises 2 sequential experimental setups. In a first set of experiments (study A), the principal feasibility of creating in vitro TEHV, which can be implanted by transapical, minimally-invasive delivery with adequate in vivo functionality, was investigated. A second set of experiments (study B) was focused on the feasibility to utilize autologous stem cells (bone marrow, peripheral blood) as a less invasive, multipotent alternative cell source. Both concepts were tested in sheep, representing the most used animal model for heart valve prostheses.

Experimental study A: merging TEHV and minimally-invasive implantation technologies, in vitro and in vivo feasibility. Valve replacements (n = 16) for minimally-invasive implantation technologies were manufactured by integrating TEHV into self-expandable stents (Figs. 1A and 1B). The TEHV were cultured in vitro using dynamic in vitro culture protocols. In vivo performance up to 8 weeks of TEHV was evaluated after transapical implantation in sheep (n = 6) in pulmonary position. Next to each im-

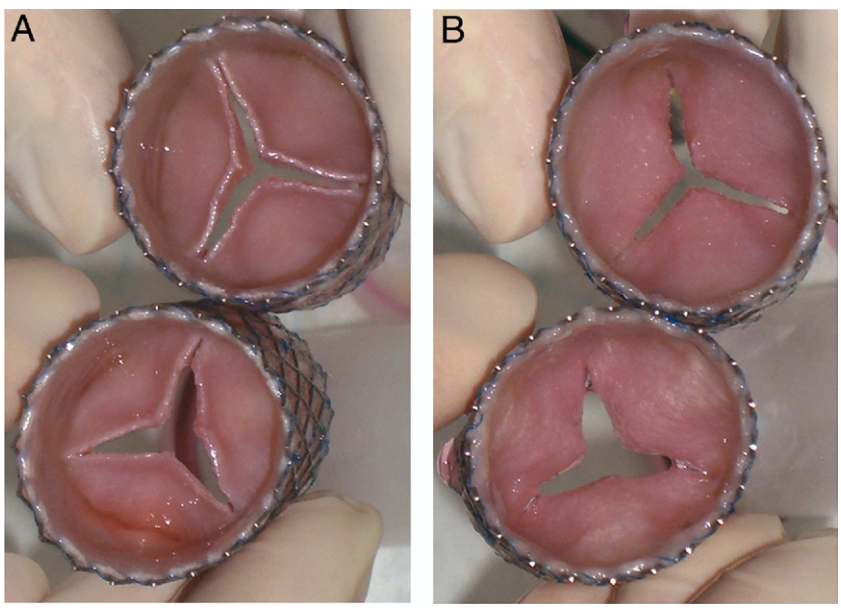

Figure 1 Macroscopic Appearance of TEHV Before Implantation

Macroscopic picture of autologous tissue engineered heart valve (TEHV) based on vascular-derived cells integrated into a self-expanding nitinol stent, **(A)** distal view and **(B)** proximal view.

planted TEHV, an additional valve was engineered for each animal to serve as a control valve (n = 6). The transapical delivery procedure required crimping of the TEHV to decrease its diameter. As a pre-condition for in vivo functionality, preservation of structural integrity and functionality after the delivery procedure was evaluated in vitro in TEHV from a single source of ovine cells (n = 4).

CELL HARVEST. Autologous myofibroblasts were harvested from ovine jugular veins as previously described (13). Endothelial cells were harvested from ovine carotid arteries. The myofibroblasts were expanded in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% lamb serum (Gibco, Invitrogen, Carlsbad, California), 1% GlutaMax (Gibco), and 0.1% gentamycin (Biochrom, Berlin, Germany). The endothelial cells were cultured in endothelial basal medium (EBM-2, complemented with 0.1% vascular endothelial growth factor, 0.4% human fibroblasts growth factor, 0.1% human recombinant long–insulin-like growth factor-1, 0.1% human epidermal growth factor, 0.1% gentamycin and amphotericin, 0.04% hydrocortisone, 0.1% heparin, 0.1% ascorbic-acid [Cambrex, Verviers, Belgium]) supplemented with 10% lamb serum. All animals received humane care, and the study was approved by the ethics committee (IMM Recherche, Paris, France) and was in compliance with the "Guide for the Care and Use of Laboratory Animals," published by the National Institutes of Health (NIH publication no. 85-23, revised 1985).

HEART VALVE SCAFFOLD FABRICATION. Trileaflet heart valve scaffolds (n = 16), were fabricated from nonwoven polyglycolic acid meshes (thickness 1.0 mm; specific gravity 70 mg/cm^3; Cellon, Bereldange, Luxembourg), coated with 1.75% poly-4-hydroxybutyrate (MW: 1 × 10^6; Tepha, Lexington, Massachusetts) by dipping into a tetrahydrofuran solution (Fluka, Steinheim, Germany). After solvent evaporation, physical bonding of adjacent fibers and continuous coating was achieved. Poly-4-hydroxybutyrate is a biologically derived rapidly degradable biopolymer, which besides being strong and pliable, is thermoplastic (61°C), and can be molded into 3-dimensional shapes. From the polyglycolic acid/poly-4-hydroxybutyrate composite scaffold material, the heart valve scaffolds were fabricated by using a heat-application welding technique. Thereafter, the scaffolds were integrated into radially self-expandable nitinol stents (length = 38 mm, outer diameter [OD] = 30 mm when fully expanded at 37°C; pfm AG, Köln, Germany) by attaching the scaffold matrix to the inner surface of the nitinol stent wires using single interrupted sutures (5-0 Prolene, Ethicon, Somerville, New Jersey). After vacuum drying overnight, the scaffolds were placed in 70% ethanol (Sigma, St. Louis, Missouri) for 4 h to obtain sterility. The ethanol was allowed to evaporate overnight, the scaffolds were washed in PBS (Sigma) and incubated overnight in medium DMEM Advanced, supplemented with 2.5% lamb serum, 1% GlutaMax, 0.3% gentamycin, and additional L-ascorbic acid 2-phosphate (0.25 mg/ml, Sigma), referred to as TE-medium, to facilitate cell attachment by deposition of proteins.

CELL SEEDING AND IN VITRO CULTURING OF TEHV. Seeding of the myofibroblasts onto the stented heart valve scaffolds (1.5 ± 0.04 × 10^6 cells/cm^2) was performed using fibrin as cell carrier (14). After seeding, the scaffolds were placed into pulse duplicator systems, exposing the leaflets to increasing dynamic strains, as previously described (15). Medium was replaced every 2 to 3 days. After 11 to 12 days, the TEHV were seeded with endothelial cells (1.6 ± 0.2 × 10^5 cells/cm^2) and cultured for 2 to 3 additional days (n = 12). For optimized surface coverage of the free edges, a subgroup of TEHV (n = 4) was cultured for 6 additional days under the same dynamic straining and in addition flow conditioning (nonpulsatile, low-pressure flow).

TEHV DELIVERY PROCEDURES. After the in vitro culture period, the OD was decreased from 30 mm to 10 mm (Fig. 2A to 2D) using a custom-built tubular-shaped crimping and delivery system (Acrostak, Geneva, Swit-

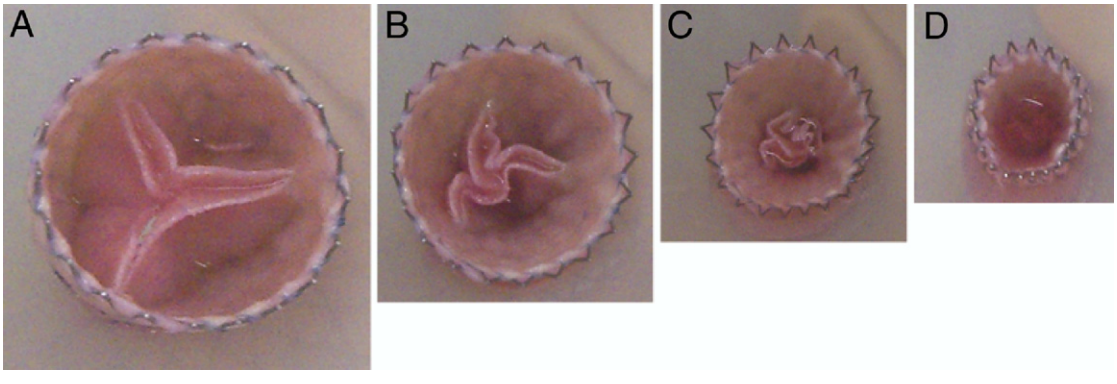

Figure 2 Crimping of TEHV for Transapical Delivery

The diameter was gradually reduced by a factor of 2.5 **(A to D)** before the tissue engineered heart valve (TEHV) was introduced into the implantation device.

zerland). The TEHV were left in the device for 30 min, representing a clinically relevant time frame. Thereafter, the TEHV were either implanted (n = 6) or directly analyzed (n = 5; 1 sample was excluded because of technical preservation problems).

IN VITRO VALVE TESTING. The in vitro valve performance of a TEHV after simulated delivery procedure was visualized in a custom-built valve tester (16), using a high-speed video camera (Phantom version 9.0, Vision Research, Wayne, New Jersey). The TEHV was exposed to physiological systemic flows for 20 min, and the resulting pressure and flow profiles were measured using flow (Transonic Systems, Ithaca, New York) and pressure sensors (Becton Dickinson, Erembodegem, Belgium). The amount of regurgitation, the effective orifice area, and the mean and maximal systolic pressure gradients were calculated and averaged over 5 cycles.

TEHV IMPLANTATION AND IN VIVO FUNCTIONALITY. For evaluation of in vivo functionality TEHV were minimally-invasively delivered into the pulmonary valve position using an anterolateral-thoracic access and antegrade approach. The valves were crimped and loaded onto a custom-made inducing system (OD = 12 mm) consisting of a rigid tube and pusher. After minithoracotomy and after pericardiotomy, the right ventricle was punctured using needle through pursestring sutures. Subsequently, the inducing system was inserted, and the valve delivered into the pulmonary artery under visualization by fluoroscopy. After the optimal position for the pulmonary heart valve replacement was defined by angiography, the valve was pushed out of the delivery system and allowed to open (Figs. 3A and 3B). Thereby, the radial forces of the nitinol stent pressed the native heart valve leaflets to the artery wall and kept them fixed. Afterward, the appropriate position and functionality of the implanted valve was visualized by angiography. Moreover, the in vivo functionality was monitored using transthoracic echocardiography during the procedure, immediately after implantation, and then weekly up to 8 weeks. Anticoagulation therapy was maintained as in conventional bioprosthetic heart valves for 7 days. The animals were sacrificed, and the TEHV were explanted after 4 weeks (n = 3) and 7 to 8 weeks (n = 3).

QUALITATIVE TISSUE ANALYSES. The TEHV (n = 16), either directly or after having functioned as implant, were evaluated macroscopically, and tissue composition was analyzed qualitatively by immunohistology and compared with native valve leaflets. The tissue sections were studied by hematoxylin and eosin staining for general tissue morphology, Masson-Trichrome staining for deposition of collagen, and Elastin van Gieson staining for detection of collagen and elastic fibers. Additionally, immunohistology was performed using the Ventana Benchmark automated staining system (Ventana Medical Systems, Oro Valley, Arizona) and antibodies for α-smooth muscle actin (α-SMA [clone 1A4, Sigma]) and von Willebrand factor (affinity purified rabbit antibodies, DakoCytomation, Copenhagen, Denmark). Primary antibodies were detected with the Ventana iVIEW DAB detection kit, yielding a brown reaction

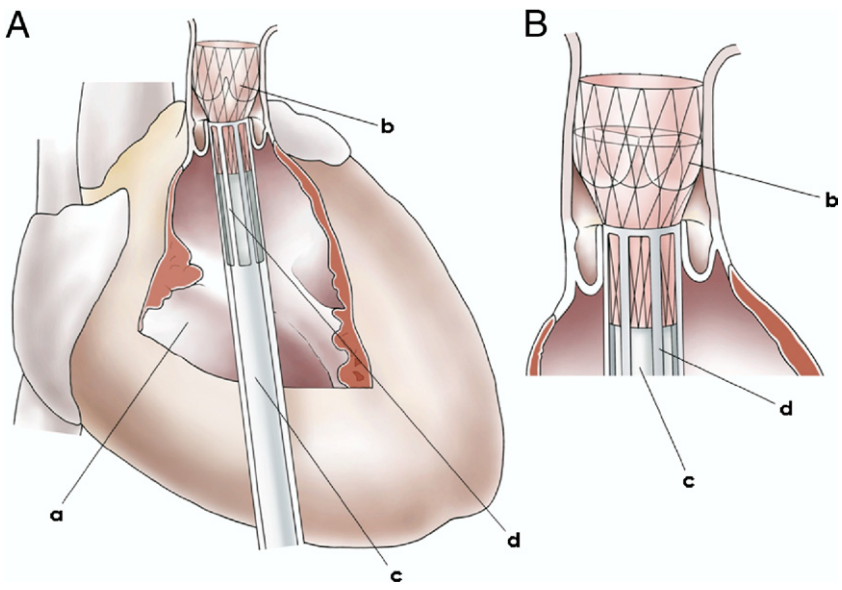

Figure 3 Delivery of the TEHV

Schematic view of transapical valve delivery **(A)** and positioning **(B)** in the pulmonary position. The delivery system loaded with the crimped tissue engineered heart valve (TEHV) is inserted transapically into the right ventricle **(a)**. The delivery system consists of an inner pusher **(c)** and a sheath **(d)**. The crimped TEHV integrated into the nitinol stent **(b)** is carefully deployed into the pulmonary position by slowly advancing the inner pusher **(c)** into the sheath system **(d)**.

product. Furthermore, tissue samples were fixed in 2% glutaraldehyde (Sigma) and sputtered with platin to examine the surface structure by scanning electron microscopy.

QUANTITATIVE TISSUE ANALYSES. The TEHV used to study the effect of the delivery procedure (n = 2 uncrimped, and n = 2 crimped), the explants (n = 6) and their corresponding control valves and native ovine valves (n = 3) were analyzed by biochemical assays for total deoxyribonucleic acid (DNA) content, as an indicator for cell number, glycosaminoglycans (GAG) content, and hydroxyproline content, as an indicator for collagen. For measuring DNA amount, the Hoechst dye method (17) was used with a standard curve prepared from calf thymus DNA (Sigma) in addition to the samples. The GAG content was determined using a modified version of the protocol described by Farndale et al. (18), and a standard curve was prepared from chondroitin sulphate from shark cartilage (Sigma) in addition to the samples. Hydroxyproline was determined with a modified version of the protocol provided by Huszar et al. (19), and a standard curve was prepared in addition to the samples.

MECHANICAL PROPERTIES. The mechanical properties of the leaflets were determined in circumferential and radial direction by uniaxial tensile tests for the TEHV used to study the effect of the delivery procedure (n = 2 uncrimped, and n = 2 crimped). Furthermore, in the explants, and the corresponding control valves, thickness of the samples was determined with a PLμ 2300 optical imaging profiler (Sensofar-Tech S.L., Barcelona, Spain). Stress-strain curves were obtained using a uniaxial tensile tester (Kammrath & Weiss GmbH, Dortmund, Germany; load cell of 20N) with a constant strain rate of 1.7%/s. The ultimate tensile stress (UTS) was determined from the curves. The modulus of elasticity (further referred to as modulus) was obtained from the slope of the linear section of the stress-strain curve as an indicator for tissue stiffness.

Experimental study B: autologous stem cells as less invasive, multipotent cell source for minimally-invasive TEHV. In the second set of experiments, the focus was on adult stem cells as autologous, noninvasive cell source. The principal experimental design was comparable to study A. However, to accommodate the technology to this alternative cell source, the cell harvest methodology and features as to biodegradable scaffold material and in vitro culture protocols have been modified as follows.

SANDWICH-STRUCTURED HEART VALVE SCAFFOLDS. These scaffolds were fabricated from biodegradable P(L,DL)LA [Poly(L-lactide-co-D,L-lactide)] multifilament fibers using a 3-dimensional valve-shaped cast and thermal fixation. Thereafter, the surfaces were coated with P(L,DL)LA based nanofibers by electrospinning in order to produce a multilayer structure mimicking the architecture of native heart valves. The P(L,DL)LA was dissolved in 1,1,1,3,3,3-hexafluoro-2-isopropanol and the polymer solution was electrospun onto the surfaces of the molded structure. The resulting sandwich-structured heart valve scaffolds were allowed to dry and were then sterilized by gamma irradiation afterward. Finally, the scaffolds were integrated into self-expanding nitinol stents (OD = 30 mm), as in experimental study A.

CELL HARVEST, ISOLATION, AND SEEDING. Adult stem cells were obtained from bone marrow as well as peripheral blood of adult sheep (INRA Brouëssyand; ethical approval no. Zurich-08-04, Switzerland). For the isolation of myofibroblast-like cells, 20 ml bone marrow was aspirated from the iliac crest, and mononuclear cells were isolated using density gradient centrifugation (Histopaque-1077, Sigma) (20). Cells were collected from the interphase and cultured in DMEM (as described in study A) under humidified incubator conditions (CO_2 5%, 37°C). Endothelial progenitor cells were collected from 20 ml peripheral blood, using the same density gradient and centrifugation as described previously (21,22). Subsequently, cells were exposed to endothelial basal medium (as described for study A) to enable endothelial cell differentiation.

According to study A, sandwich-structured trileaflet heart valve scaffolds (n = 4) were seeded with bone marrow-derived myofibroblast-like cells using fibrin as a cell carrier (14) and cultured under static conditions. After 6 days, the heart valves were inserted into a flow-bioreactor system (23) and exposed to pulsatile flow for 3 additional days followed by endothelialization using autologous peripheral blood-derived endothelial cells. After seeding, the constructs were kept under static conditions for 3 additional days to enable endothelial cell attachment and proliferation on the valve surfaces.

TEHV IMPLANTATION, IN VIVO FUNCTIONALITY, AND ANALYSES. In vivo performance up to 4 weeks of TEHV (n = 2) was evaluated in pulmonary position. Next to each implanted TEHV, an additional valve was engineered for each animal to serve as a control valve (n = 2). Minimally-invasive implantation procedures, assessment of TEHV functionality and tissue analysis methodology was performed as in the experimental study A. In addition to the immunohistology of study A, tissue sections were also stained for endothelial nitric oxide synthase (eNOS) type III (affinity purified rabbit antibodies; BD Biotechnology, Santa Cruz, California) with signal enhancement using the Ventana amplification kit.

Statistical analysis of study A and study B. Quantitative data are presented as mean ± SD of the mean. Student *t* tests were used to determine the influence of the simulated delivery procedure on TEHV properties, with each leaflet being considered as n = 1. The use of the leaflets as the unit of analysis was made without adjustment for correlated observations within individuals. All p values <0.05 were considered statistically significant. The intersheep variability did not allow for comparisons between time points. Furthermore, results of vascular cell- and stem cell-based

TEHV were not directly comparable because of methodological differences in fabrication.

Results

Impact of crimping on living TEHV. The impact of crimping on the living TEHV was investigated in valves generated from vascular-derived cells (study A). The TEHV showed proper opening and closing behavior after simulation of the delivery procedure when exposed to systemic conditions, with a regurgitation value of 8 ± 3%. The effective orifice area during systole was 1.9 ± 0.1 cm^2, and the mean and maximal pressure gradients during diastole were 5.1 ± 0.3 mm Hg and 17.6 ± 0.3 mm Hg, respectively (thereby fulfilling the International Organization for Standardization 5840 guidelines for valvular replacements of 29 mm).

Cellular tissue formation and abundant amounts of collagen were demonstrated in the leaflets (Figs. 4A and 4B) and the wall, with higher cellularity and collagen deposition in the outer tissue layers. Elastin was not detected. Within experimental study A, no significant differences were found in vitro and in vivo with regard to extracellular matrix composition, morphological structure, and mechanical properties in relation to the adapted in vitro conditioning protocol (that is subgroup including additional flow conditioning).

After the delivery procedure, preserved cellularity, structure, and collagen deposition was demonstrated in the leaflets (Figs. 4E to 4H) and the wall. Endothelial cells, covering the luminal surfaces of the leaflets (Fig. 4C), were, although incomplete, detectable after the simulated delivery procedure (Fig. 4G). Scanning electron microscopy pictures of the hinge region and the leaflets, without simulated delivery (Figs. 5A and 5C) and with simulated delivery (Figs. 5B and 5D) confirmed the preservation of structural integrity after the delivery procedure.

Moreover, the delivery procedure induced no significant tissue damage resulting in cell loss as indicated by the

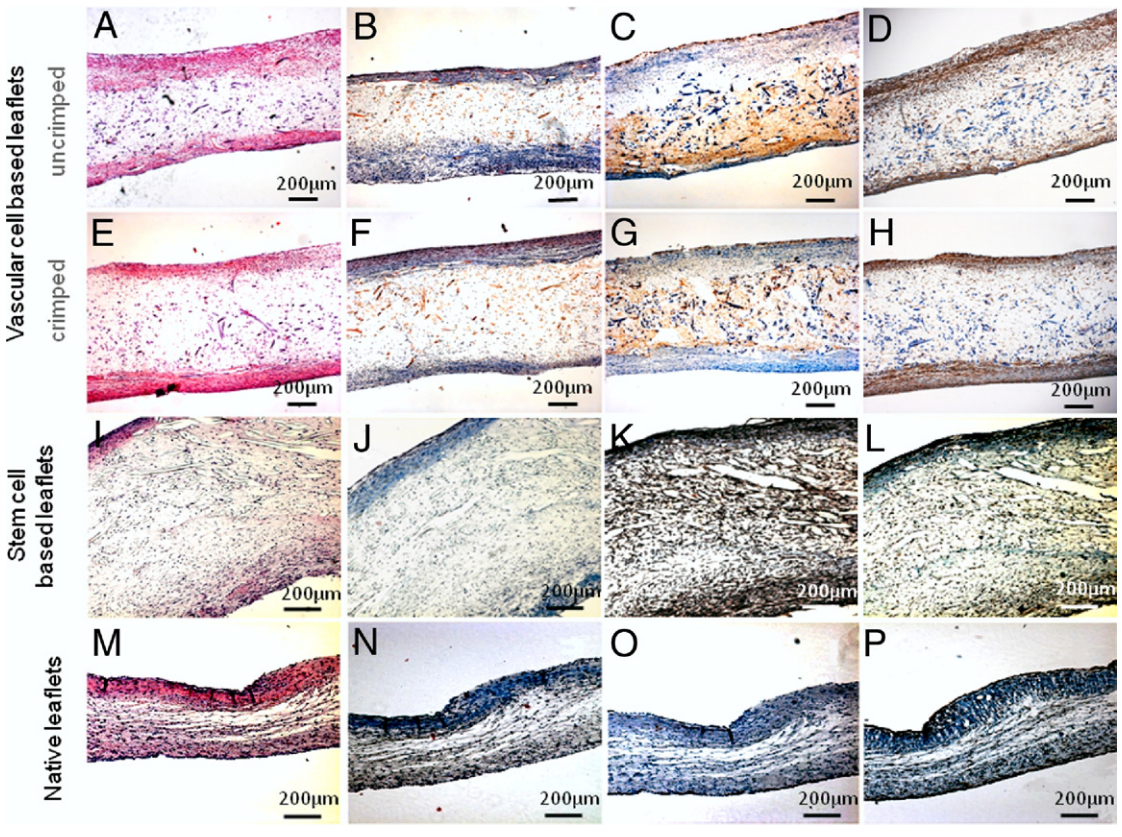

Figure 4 Histology of TEHV Leaflets on Basis of Vascular-Derived Cells, Uncrimped and Crimped

Hematoxylin and eosin stain **(A, E)**, Masson's Trichrome stain **(B, F)**, von Willebrand factor stain **(C, G),** and α-smooth muscle actin (SMA) staining **(D, H)**. Before simulated delivery, cellular tissues were demonstrated **(A)** with abundant amounts of collagen in the outer layers **(B)** and endothelial coverage **(C)** at the luminal side of the leaflets. After simulated delivery, the leaflets demonstrated preserved structural integrity **(E to H)**. However, incomplete endothelialization of the tissue engineered heart valve (TEHV) was detected **(G)**. Stem cell-derived TEHV leaflets **(I to L)**: hematoxylin and eosin staining demonstrated a tissue architecture for adult stem cells based leaflets **(I)** comparable to their native counterpart **(M)**. Collagen production was as visualized by Masson's Trichrome staining **(J, N)**. The α-SMA expression could be detected in stem cells **(K)** but was absent in native interstitial valve cells **(O)**. Moreover, endothelial nitric oxide synthase was expressed in the surface cells of the stem cell-based **(L)** as well as the native valvular leaflets **(P),** indicating the presence of an endothelial layer.

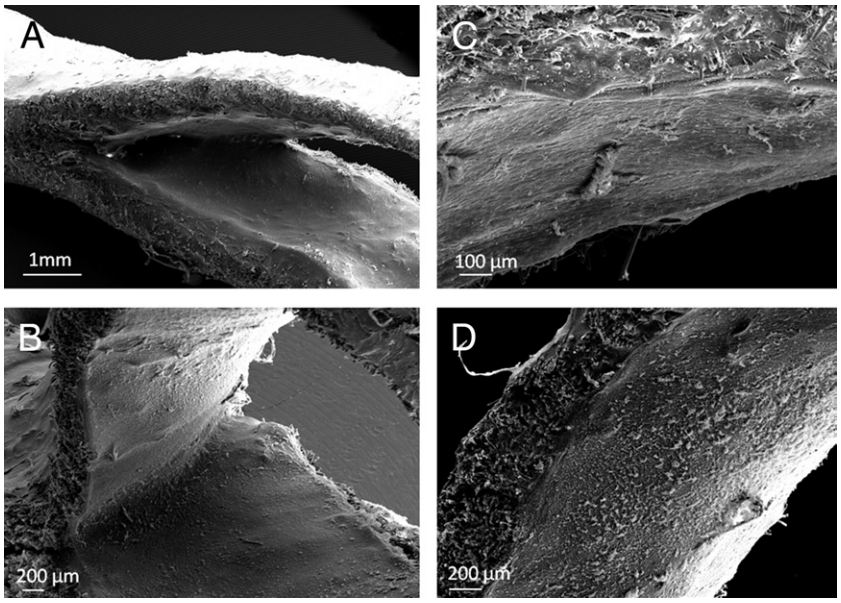

Figure 5 SEM of TEHV Leaflets Before and After Simulated Delivery Procedure

Scanning electron microscopy (SEM) of tissue engineered heart valve (TEHV) leaflets, showing the hinge region **(A, B)** and the leaflets **(C, D)**, demonstrated preserved tissue structure after simulated delivery **(B, D)**.

amount of DNA (3.4 ± 0.2 μg/mg uncrimped and 3.0 ± 0.4 μg/mg crimped), GAG (12.3 ± 0.8 μg/mg uncrimped and 11.4 ± 1.2 μg/mg crimped), and hydroxyproline (9.7 ± 0.6 μg/mg uncrimped and 9.7 ± 0.5 μg/mg crimped). The amount of DNA, GAG, and hydroxyproline of the TEHV was lower compared with the amounts measured in native ovine leaflets (29%, 29%, and 17%, respectively). When normalized to the amount of DNA, it was noted that the extracellular matrix synthesis activity in the TEHV was closer or even comparable to that in native ovine leaflets (hydroxyproline per DNA 61% and GAG per DNA 100%).

Comparable mechanical behavior was found for the radial and circumferential strips of the leaflets in both the crimped and uncrimped TEHV and further treated as 1 group. The delivery procedure did not affect the modulus and UTS (Table 1).

In vivo performance of TEHV. The transapical implantation procedures of study A and study B were uneventful in all animals (n = 8) regarding perioperative morbidity or mortality. Furthermore, all valves were deployed successfully at the targeted site, demonstrated by fluoroscopy (Online Video 1, video fluoroscopy). No migration of the TEHV or paravalvular leakage was observed. Proper opening and closing behavior was demonstrated by transthoracic echocardiographic measurements (Online Videos 2A and 2B). The intraoperative transthoracic echocardiogram revealed an initial mean pressure gradient of 30 mm Hg, constantly decreasing down to 10 mm Hg over the whole period of implantation time. Minimal regurgitation was detected in 2 animals, which remained unchanged over the whole period of implantation time.

Macroscopic analysis of all explanted valve leaflets, demonstrated thickened tissue formation (Fig. 6). The wall of the TEHV was integrated into the adjacent tissue. Dense, layered tissue with thickened outer layers, containing abundant amounts of collagen, was demonstrated (Figs. 7B and 7E). No elastin was detected. Cells staining positive for α-SMA were identified mainly in the wall of the explanted valves, but also in the middle of the leaflets (Figs. 7D and 7G). In some areas, a deposition of fibrinous material was detected.

Quantitative tissue analyses for the amounts of DNA, GAG, and hydroxyproline of all explanted valve leaflets, expressed in percentages to native valve leaflets, are summarized in Table 2. Note that the quantitative results of the

Table 1 Biomechanical Properties of Crimped, Uncrimped, and Explanted Vascular Cell- and Stem Cell-Based TEHV

	Modulus (MPa)	UTS (MPa)
Vascular cell-based TEHV		
Crimped TEHV (n = 2)	3.09 ± 0.49	0.60 ± 0.13
Uncrimped TEHV (n = 2)	3.08 ± 0.56	0.54 ± 0.08
Explanted TEHV, 4 weeks (n = 3)	0.83 ± 0.70	0.27 ± 0.22
Explanted TEHV, 8 weeks (n = 3)	2.20 ± 4.10	1.01 ± 2.17
Stem cell-based*		
Explanted TEHV – 4 weeks (n = 2)	0.63 ± 0.15	0.27 ± 0.06

Mean values and standard deviations for modulus (indicating elasticity) and ultimate tensile strength (UTS) in megapascal (MPa). *Results of adult stem cell-based tissue engineered heart valve (TEHV) are not directly comparable to vascular cell-based TEHV because of the methodological differences in fabrication.

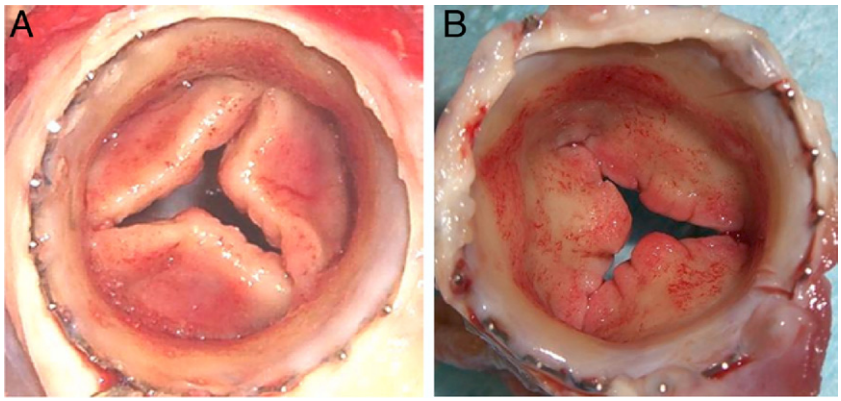

Figure 6 **Macroscopic Appearance of Explanted TEHV**

Representative photographs of the leaflets and wall of an explanted tissue engineered heart valve (TEHV) after 4 weeks, **(A)** distal view and **(B)** proximal view.

stem cell-based TEHV (experimental study B) are not directly comparable to the vascular cell-based TEHV because of the principal methodological difference between studies A and B (see also the Methods section). The amounts of DNA, GAG, and hydroxyproline present in the explants appeared equal or increased when compared with their corresponding controls for the vascular cell-based TEHV.

Autologous stem cell-based minimally-invasive TEHV. Adult stem cells were isolated from bone marrow as well as peripheral blood and demonstrated growth characteristics comparable to those of vascular cells, resulting in sufficient quantity for seeding at passage 3. Bone marrow-derived cells cultured in fibroblast-inducing medium showed a myofibroblast-like phenotype expressing α-SMA. Peripheral blood-derived endothelial progenitor cells exposed to endothelial medium formed cobblestone-like monolayers and showed endothelial characteristics similar to previous studies (10,22).

After the seeding and in vitro culture process, all valves macroscopically demonstrated excellent tissue formation. Histology of valves revealed cell attachment and ingrowth as observed in hematoxylin and eosin staining (Fig. 4I). Compared to cross-sections of native heart valves (Fig. 4M), engineered leaflets were thicker than their native counterparts, similar to the vascular-cell based TEHV, due to the initial thickness of the scaffold material. Collagen fibers were predominantly present in the outer layers as visualized by Masson's Trichrome staining (Fig. 4J) and comparable to the distribution pattern in native leaflets (Fig. 4N). Cells in the newly formed tissues expressed α-SMA (Figs. 4D, 4H, and 4K), whereas native interstitial valve cells did not (Fig. 4O). On the surfaces of the TEHV leaflets, eNOS expression could be detected as well as on the native valve leaflets, indicating the presence of an endothelial layer (Figs. 4L and 4P).

Valve functionality with leaflet movement and sufficient opening and closing behavior was observed after implantation. No paravalvular leakage could be detected in the follow-up controls.

Hematoxylin and eosin staining of explants demonstrated layered tissues without indication for the foreign body reaction. An example of an explanted leaflet based on adult stem cells is shown in Figure 7A. Masson's Trichrome staining confirmed the presence of collagen predominantly in the outer layers (Fig. 7B). Cells of the leaflet surface layer expressed apart from eNOS (Fig. 7C) also α-SMA (Fig. 7D), resembling the staining pattern of native valve endothelial cells. Moreover, α-SMA expression could be detected in interstitial cells of the newly formed tissue and was lower in explanted TEHV leaflets. The modulus of the valve leaflets was decreased after 4 weeks in vivo.

Discussion

Minimally-invasive techniques have recently shown to be promising alternatives to conventional heart valve surgery (2,24,25). However, because the currently used replacement materials are bioprosthetic, it is likely that they are associated with calcification (26), leading to dysfunctionality as has been observed for conventional valves (27). Living autologous TEHV, based on rapidly degrading polymer scaffolds, and minimally-invasive valve replacement procedures represent promising technologies for patients with valvular heart disease.

This paper presents the first "proof of principle" in vivo data with regard to the combination of the novel technologies tissue engineering including stem cells and minimally-invasive implantation. In particular, the use of autologous stem cells might enable a truly minimally-invasive autologous valve replacement, from cell harvest to implantation. In summary, autologous TEHV, integrated into self-expandable stents, were developed and tested in vitro for

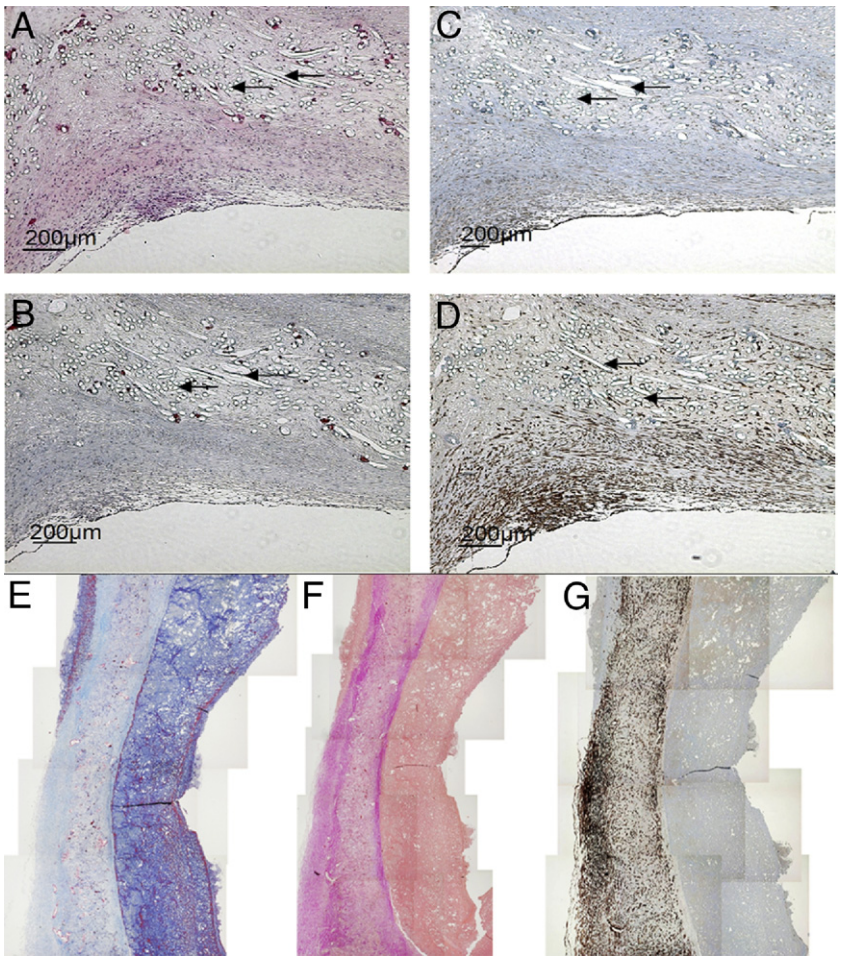

Figure 7 **Histology of Explanted TEHV Leaflets on Basis of Adult Stem Cells and Vascular-Derived Cells**

Adult stem cell-based tissue engineered heart valve (TEHV): the explanted tissues showed layered tissue architecture visualized by hematoxylin and eosin staining **(A)**. In Masson's Trichrome staining, collagen fibers were predominantly found in the outer tissue layer **(B)**. Cells of the surface layer expressed endothelial nitric oxide synthase **(C)** and α-smooth muscle actin (SMA) **(D)**. Moreover, α-SMA expression could be detected in subpopulations of the inner layer **(D)**. **Arrows** indicate reminiscent scaffold material. Vascular cell-based TEHV: a compilation of several pictures is combined to a microscopical map of part of a TEHV leaflet. These pictures show layered tissue formation with abundant amounts of collagen **(E)** and no elastin **(F)**. Active α-SMA expressing myofibroblasts were located only in the middle layer of the leaflets **(G)**.

preservation of structural integrity after the delivery procedure before they were transapically implanted in the pulmonary position of sheep. Delivery and deployment of the TEHV were demonstrated to be safe as all procedures were uneventful. The living TEHV demonstrated adequate functionality, but thickened leaflets.

The application of the TEHV for transapical delivery required the valve to be integrated into a stent. As a clinically established concept, nitinol stents were chosen as they possess shape memory, are self-expanding, and in contrast to balloon-expandable stents, allow the least destructive expansion of the delicate living TEHV. The heart valve design included a vascular wall for prevention of paravalvular leakage and to protect the leaflets from damage by the stent struts during the crimping and delivery procedure (28). Aiming at an autologous approach, vascular cells were utilized as they have proven to be successful for heart valve tissue engineering (8,13). The transapical delivery required crimping of the TEHV by a ratio of approximately 2.5 compared with the original diameter, which is similar to the crimping ratio clinically in stented bioprosthetic valves (2). In vitro simulation of the delivery procedure revealed proper valve functioning and preserved structural integrity and no detectable damage to the leaflets and the wall. Although incomplete endothelialization of the TEHV was seen as a possible indicator of surface friction, in the absence of any thromboembolic event, this appeared to be of no clinical relevance. The simulation of the delivery procedure did not alter the mechanical properties as determined by tensile tests.

Morphological analyses of the explanted leaflets based on autologous vascular cells demonstrated thickened and lay-

Table 2 Quantitative Tissue Analysis of Explanted TEHV on Basis of Vascular-Derived Cells and Stem Cells

	DNA (% Native Leaflets)	GAG (% Native Leaflets)	Hyp (% Native Leaflets)
Vascular cell-based TEHV			
4 weeks (n = 3)	49 ± 24	39 ± 9	15 ± 6
8 weeks (n = 3)	44 ± 18	39 ± 6	18 ± 3
Adult stem cell-based TEHV*			
4 weeks (n = 2)	86 ± 54	150 ± 11	26 ± 6

*Results of adult stem cell-based tissue engineered heart valves (TEHV) are not directly comparable to vascular cell-based TEHV because of the methodological differences in fabrication. Mean amounts and standard deviations of glycosaminoglycans (GAG), deoxyribonucleic acid (DNA), and hydroxyproline (Hyp) expressed in percentage to native valve leaflets.

ered tissue formation that may be part of a "natural" remodeling process, as in previous studies, the initially thickened leaflets were shown to fully remodel to thin native-like leaflets beyond 8 to 12 weeks (8). The tissue engineered wall structures were integrated into the adjacent native tissue for all explanted valves. Interestingly, it seemed that the increased extracellular matrix deposition in the explanted vascular cell-based TEHV was mainly due to GAG deposition. This observation is in accordance with early development in fetal heart valves (29), indicating that the remodeling process of the TEHV after 8 weeks may still be in an early developmental stage. The observed deposition of fibrinous material on the valve surfaces might have been induced by the compromised endothelial properties. Further in-depth studies are necessary to unravel the biological mechanisms behind the thickening of the implanted TEHV as well as the potential role of the partial lack of endothelial cells in the remodeling process.

As in previous tissue engineering studies, no relevant amounts of elastin were detectable in the engineered valve tissues by quantitative analysis. This was also reflected by the measured reduced pliability of the engineered heart valves and represents a concern with regard to the long-term functionality of the engineered valves in particular with regard to future systemic use. To address this limitation, improved in vitro protocols are under investigation specifically focusing on accelerated elastin formation and exploring various stimulation protocols, such as hypoxia (30).

Aiming at minimal invasiveness also with respect to cell harvest, preliminary in vitro and in vivo experiments with autologous living TEHV based on adult stem cells were performed in the second set of experiments. Functional heart valves were successfully fabricated from myofibroblast-like cells isolated from bone marrow aspirates and peripheral blood-derived endothelial cells seeded onto sandwich-structured scaffolds. Successful endothelialization of heart valve surfaces before implantation using peripheral blood-derived endothelial progenitor cells could be demonstrated. On the leaflet surfaces, differentiated peripheral blood-derived endothelial progenitor cells demonstrated phenotypes comparable to native valve endothelial cells as indicated by the co-expression of eNOS and α-SMA (31). These data suggest peripheral blood as a promising source for engineering native-analogous endothelia. Finally, the successful minimally-invasive implantation of these heart valves was demonstrated in this feasibility study. Thus, the use of these 2 easy-to-access adult stem cell sources (bone marrow and peripheral blood) realizes a complete minimally-invasive (nonsurgical) cell harvest protocol for autologous heart valve tissue engineering that could be easily integrated into routine ambulant clinical procedures.

Although based on the same principal concept, the presented data in fact reflect a certain evolution/progress with regard to details of the involved methodologies. In particular, the biodegradable scaffold material and design of study A (feasibility) was modified and further developed for the specific use of stem cells in study B. In contrast to a less structured (mesh structure, study A), a layered (sandwich) design was used in study B, mimicking the 3-layered structure of a natural heart valve and thereby providing a more "biologically inspired" microenvironment to the cells. This inherently implies a limitation as to direct comparisons between the 2 study groups.

Further studies are mandatory to elucidate the long-term fate of such living engineered heart valves after minimal invasive delivery as well as the underlying tissue remodeling mechanisms observed in vivo. Particularly, the role of the seeded cells with respect to their origin and state of differentiation has to be systematically assessed by, for example, cell-tracking methodology, also to validate whether seeded cells persist in neotissues and contribute to tissue remodeling (32). However, addressing these questions was beyond the scope of this pilot study and will be investigated in future experiments.

Conclusions

These first results of combining minimally-invasive valve replacement procedures with tissue engineering are promising and demonstrate the feasibility of merging the current "hot topics" in heart valve replacement technologies. Moreover, when combined with minimally-invasively harvested autologous adult stem cells, a clinically relevant complete minimally-invasive concept may be realized. Although representing only a first in vivo investigation revealing challenging questions, process safety has been demonstrated, and future studies have been initiated to assess long-term in vivo functionality as well as a completely transvascular catheter-based delivery approach.

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Key Words: heart valves ■ minimally invasive ■ stem cells ■ cells ■ tissue engineering.

APPENDIX

For supplementary Videos 1 and 2, please see the online version of this article.