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Endothelial Progenitor Cells as a Sole Source for *Ex Vivo* Seeding of Tissue-Engineered Heart Valves

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Purposes: We investigated whether circulating endothelial progenitor cells (EPCs) can be used as a cell source for the creation of a tissue-engineered heart valve (TEHV). *Methods:* Trileaflet valved conduits were fabricated using nonwoven polyglycolic acid/poly-4-hydroxybutyrate polymer. Ovine peripheral blood EPCs were dynamically seeded onto a valved conduit and incubated for 7, 14, and 21 days. *Results:* Before seeding, EPCs were shown to express CD31^{+}, eNOS^{+}, and VE-Cadherin^{+} but not α-smooth muscle actin. Histological analysis demonstrated relatively homogenous cellular ingrowth throughout the valved conduit. TEHV constructs revealed the presence of endothelial cell (EC) markers and α-smooth muscle actin^{+} cells comparable with native valves. Protein levels were comparable with native valves and exceeded those in unseeded controls. EPC-TEHV demonstrated a temporal pattern of matrix metalloproteinases-2/9 expression and tissue inhibitors of metalloproteinase activities comparable to that of native valves. Mechanical properties of EPC-TEHV demonstrated significantly greater stiffness than that of the unseeded scaffolds and native valves. *Conclusions:* Circulating EPC appears to have the potential to provide both interstitial and endothelial functions and could potentially serve as a single-cell source for construction of autologous heart valves.

Introduction

APPROXIMATELY 20,000 infants are born with congenital heart disease in the United States each year. Repair of the congenital cardiac defects is now possible; however, numerous defects involve malformations or absence of the pulmonary valve (PV) and main pulmonary arteries, complicating the repair.[1] Although clinically approved homograft valves are acceptable, they cannot grow with pediatric patients requiring reoperations. These shortcomings have motivated the exploration of tissue engineering (TE) cardiac valves and conduit arteries by seeding autologous cells onto the bioabsorbable synthetic scaffolds or decellularized xenogenic tissues. A less invasive cell source for tissue-engineered heart valve (TEHV) would be preferable to avoid the sacrifice of systemic blood vessels.

We demonstrated previously that ovine blood-derived endothelial progenitor cells (EPCs) could be used to en-dothelialize small diameter vascular grafts with sustained patency and vascular function.[2] Importantly, ovine EPCs have been shown to transdifferentiate from an endothelial to mesenchymal phenotype in response to transforming growth factor.[3,4] This transdifferentiation, reminiscent of the endothelial–mesenchymal transformation (EMT) that occurs in the endocardial cushions during valve development, can also be induced in human aortic valve–derived EC.[5,6] These studies suggest that EPC may be uniquely suited for creating TEHV. Additional studies have demonstrated that ovine EPC differentiate into mesenchymal cells, as indicated by the induction of α-smooth muscle actin (α-SMA) when seeded onto the TE scaffolds.[7]

The current report investigates whether circulating EPC (cEPC) could potentially provide endothelial and interstitial cell functions and produce sufficient extracellular matrix (ECM) within the biodegradable scaffold environment to produce a TEHV.

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Materials and Methods

Animal care and experimental procedures were approved by the Animal Care Committee of the Children's Hospital Boston.

Valved conduit construction

Nonwoven polyglycolic acid (PGA) polymer (thickness 1.0 mm, specific gravity 69 mg/cm^3; Albany International Research Company, Mansfield, MA) was cut into two rectangular pieces and were interlocked with a Foster needle creating a large central pocket. The scaffold was rolled into a tube to create a valved conduit and was submerged into a 1% solution (w/v) of poly-4-hydroxybutyrate (P4HB) in tetrahydrofuran (Tepha, Lexington, MA) followed by thermal bonding of the seam, and then sterilized with ethylene oxide. Final conduit dimensions measured 18 mm by 30 mm with a surface area of 20 cm^2 and thickness of 1 mm (Fig. 2).

Cell isolation and culture

EPCs, vascular EC, and smooth muscle cells were isolated and expanded from peripheral blood and carotid arteries, respectively, of juvenile sheep (age 5–10 weeks; weight 25–35 kg) as previously described.[4,7] Isolated mononuclear cells were transferred to endothelial cell growth medium-2 supplemented with growth factors and cytokines (Bulletkits®; Lonza, Walkesville, MA) without hydrocortisone, antibiotics, and 20% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) and plated. After 4 days, nonadherent cells were removed, and the culture was maintained through days 7–21.

Preparation of the TEHV

Dynamic rotational seeding and culturing were performed as previously described.[4]

Analysis of EPC cultures and TEHV

Indirect immunofluoresence. First passage cells were plated and methanol fixed as described.[4] Slides were incubated with primary antibodies against goat CD31 (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit eNOS (1:100), mouse VE-Cadherin (1:250; Abcam, Cambridge, MA), and mouse α-SMA Clone 1A4 (1:500; Dako, Carpinteria, CA). Nuclear counterstaining was performed with 4, 6-diamidino-2-phenylindole (Invitrogen, Carlsbad, CA). Slides were photographed under a fluorescence microscope (Nikon Eclipse TE2000, Nikon Instruments Inc., Melville, NY). Isotype-matched IgG, ovine vascular EC, and vascular smooth muscle cell were used as controls.

Histology and immunohistochemistry. Histological analysis and characterization of cell phenotypes were performed as previously described.[4,7,8] Representative portions of TEHV and native PV (adult sheep 4–8 months) were formalin fixed and paraffin embedded. Serial sections (6 μm) were stained with hematoxylin and eosin for morphology and antibodies specific for mouse vascular endothelial growth factor (VEGF)-R2 (1:20; Santa Cruz Biotechnology), rabbit Laminin (LM) (1:100; Chemicon, Temecula, CA), Fi-

bronectin (FN) and collagen types III (Coll III) (1:40, 1:200, respectively; Abcam), mouse CD31 and collagen type I (Coll I) (1:25 and 1:100, respectively; Abcam) rabbit vWF (1:100) (Dako), mouse α-SMA Clone 1A4 (1:120), and rabbit elastin (EL) (1:50; Sigma-Aldrich, St. Louis, MO).

Immunoblotting. Conduit extracts ($n = 4$) were lysed, processed, and immunoblotted as previously described.[4,7,9] Membranes were incubated with primary antibodies specific for goat CD31 (1:500; Santa Cruz Biotechnology), mouse α-SMA Clone 1A4 (1:1000), and α-mouse Tubulin Clone DM 1A (1:500) (Sigma-Aldrich). Detection of ECM proteins was performed using polyclonal antibodies: rabbit anti-human Coll I and III and FN (1:2500, 1:2500, and 1:5000, respectively; Abcam), LM (1:5000; Millipore Corporation, Billerica, MA), TE (1:1000; Elastin Products Company, Inc., Owensville, MO), and mouse monoclonal EL Clone BA-4 (1:500; Sigma-Aldrich).

Quantitative biochemical matrix analysis. DNA, collagen, and sulfated glycosaminoglycans (S-GAG) were quantified as previously described.[10] Samples were extracted, and DNA (Picogreen dsDNA Quantitation Kit; Molecular Probes, Inc., Eugene, OR), collagen, and S-GAG (Sircol™ and Blyscan™ assay kits; Biocolor Ltd, County Antrim, United Kingdom) were measured.

Determination of matrix metalloproteinase and tissue inhibitors of metalloproteinase activities in TEHV. Extraction and detection of matrix metalloproteinases (MMP-2 and -9, respectively)/gelatinases A and B and tissue inhibitors of metalloproteinase (TIMP) activities were performed as described previously.[11,12] Conduits were processed in extraction buffer (2 M NaCl, 10 mM tris[hydroxymethyl]-aminomethane [Tris], pH 7.00, and 0.02% NaN$_3$). The supernatant was dialyzed against MMP/TIMP assay buffer (50 mM Tris–HCl, pH 7.6, 0.2 M NaCl, and 1 mM CaCl$_2$, pH 7.4). Conduit extracts were resolved on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis containing 4% sucrose. Gels were incubated in substrate buffer (50 mmol/LTris [pH 8], 5 mmol/L CaCl$_2$, and 0.02% NaN$_3$). Purified MMPs were included as positive controls.[12] Collagenase assay and TIMP activities were performed as previously described.[11–13] Samples were concentrated and dialyzed against collagenase assay buffer. Latent collagenase was trypsin activated. Samples were added to wells containing ^{14}C-radiolabeled type I collagen and incubated. The supernatants containing soluble radiolabeled collagen were counted (Beckman model LS-3801, Beckman Coulter, Inc., Fullerton, CA). MMP inhibitory activity was determined by the addition of 100 μL of bovine corneal collagenase to an appropriately diluted sample in wells containing ^{14}C-radiolabeled collagen and incubated. One inhibitory unit was defined as the amount of sample required to obtain half maximal inhibition of collagenase activity in 2.5 h at 37°C.

Effective stiffness measurements. The tissue effective stiffness (E) was measured as previously described.[14] Rectangular samples were marked and subjected to three-point flexure by a calibrated flexure bar of known stiffness. Displacements of the sample, flexure bar, and reference markers
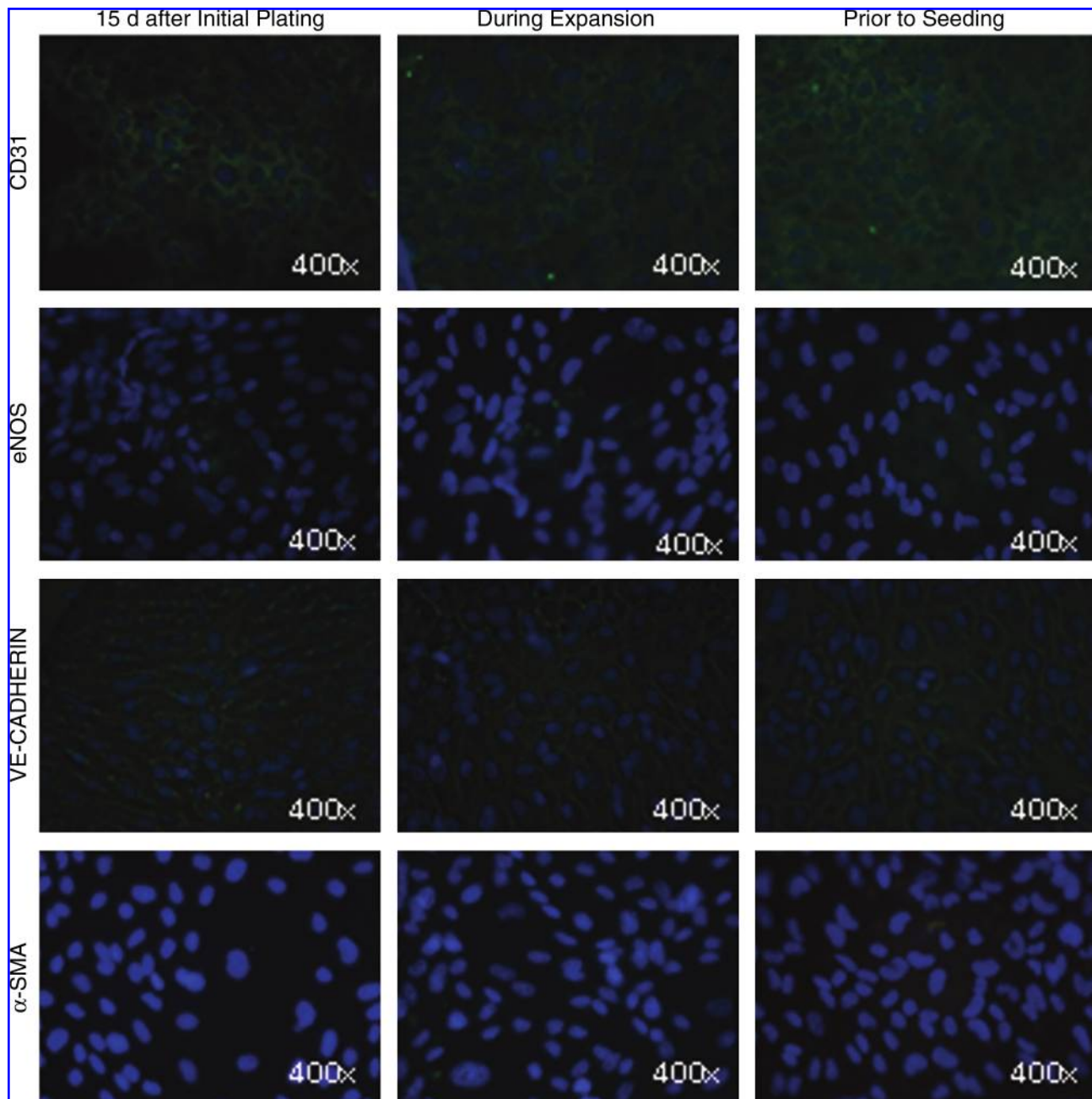
FIG. 1. Expression of endothelial cell antigens by EPC. Immunofluorescence was performed on isolated clonal and single-population–amplified pure cultures of EPC days 7–21 after blood isolation (first panel), during expansion (second panel), and before seeding (third panel) with antibodies to CD31, eNOS, VE-Cadherin, and α-SMA. Shown are antigen staining (green) and nuclei 4, 6-diamidino-2-phenylindole staining (blue). Analysis of four EPC per stage of characterization produced similar results. Original magnification, ×400. EPC, endothelial progenitor cells; α-SMA, α-smooth muscle actin.

were recorded and used to calculate the applied moment and the resulting change in curvature.

Statistical analysis

All results were expressed as mean ± standard error of the mean. Comparisons between groups were made with a Student's *t*-test (Sigma Stat; SPSS, Chicago, IL). If measurements failed the normality test, the nonparametric Mann–Whitney rank sum test was used. *p*-Values < 0.05 were considered significant.

Results

cEPC demonstrates endothelial phenotype before seeding

EPC colonies were characterized and identified: days 7–21 attached cells after initial plating and isolated clonal and single-population–amplified EPC cultures during expansion and before seeding. All adherent cells formed spindle-shaped cells that had cobblestone morphology as previously described.[4,15] Similar results were obtained for

EPC colony-forming cells regardless of whether adherent cells where isolated clonally or from single population. EPC-derived cells showed numerous endothelial cell-surface antigens including CD31, eNOS, and VE-Cadherin and stained negatively for α-SMA (Fig. 1).

Tissue formation of the EPC-TEHV in vitro

Seeded constructs ($n = 4$) after 21 days were analyzed in two parts: wall and leaflet. Figure 3a depicts the tissue analysis of TEHV. Histological examinations of the native PV (Fig. 3b) revealed three distinct layers (fibrosa, spongiosa, and ventricularis), and TEHV (Fig. 3c–f) showed the junction of the leaflet and conduit wall (Fig. 3c). Cellular architecture with cellular ingrowth throughout the scaffold was observed in the TE leaflets (Fig. 3f).

In vitro expression of mature and functional EC and α-SMA in EPC-TEHV

Immunohistochemical staining of TEHV ($n = 3$) demonstrated preferential expression of CD31$^+$ and VEGF-R2$^+$ cells on the luminal surface and expression of α-SMA on the cells in the "interstitium" of the TE conduit, whereas vWF$^+$ cells were localized throughout the valved conduits, comparable with the expression pattern in the native PV (Fig. 4A). Immunoblots of cell lysates from full-thickness samples of the wall demonstrated expression of CD31 and α-SMA (Fig. 4B).

DNA, ECM, and S-GAG contents in EPC-TEHV

After 7 and 21 days of incubation, the DNA content in TE constructs was significantly greater by 52% ($p < 0.001$) and 38% ($p < 0.05$), respectively, versus seeded constructs after 14 days (Fig. 5A). Collagen content after 7 days was significantly greater by 58% ($p < 0.05$) versus seeded constructs after 14 days. Collagen content at 21 days was increased by 24%. Significant production in collagen content (45%, $p < 0.05$) was observed at day 7 (Fig. 5B). Contrastingly, S-GAG production at 21 days was significantly greater by 60–65% ($p < 0.05$) versus seeded constructs after 7 and 14 days (Fig. 5C).
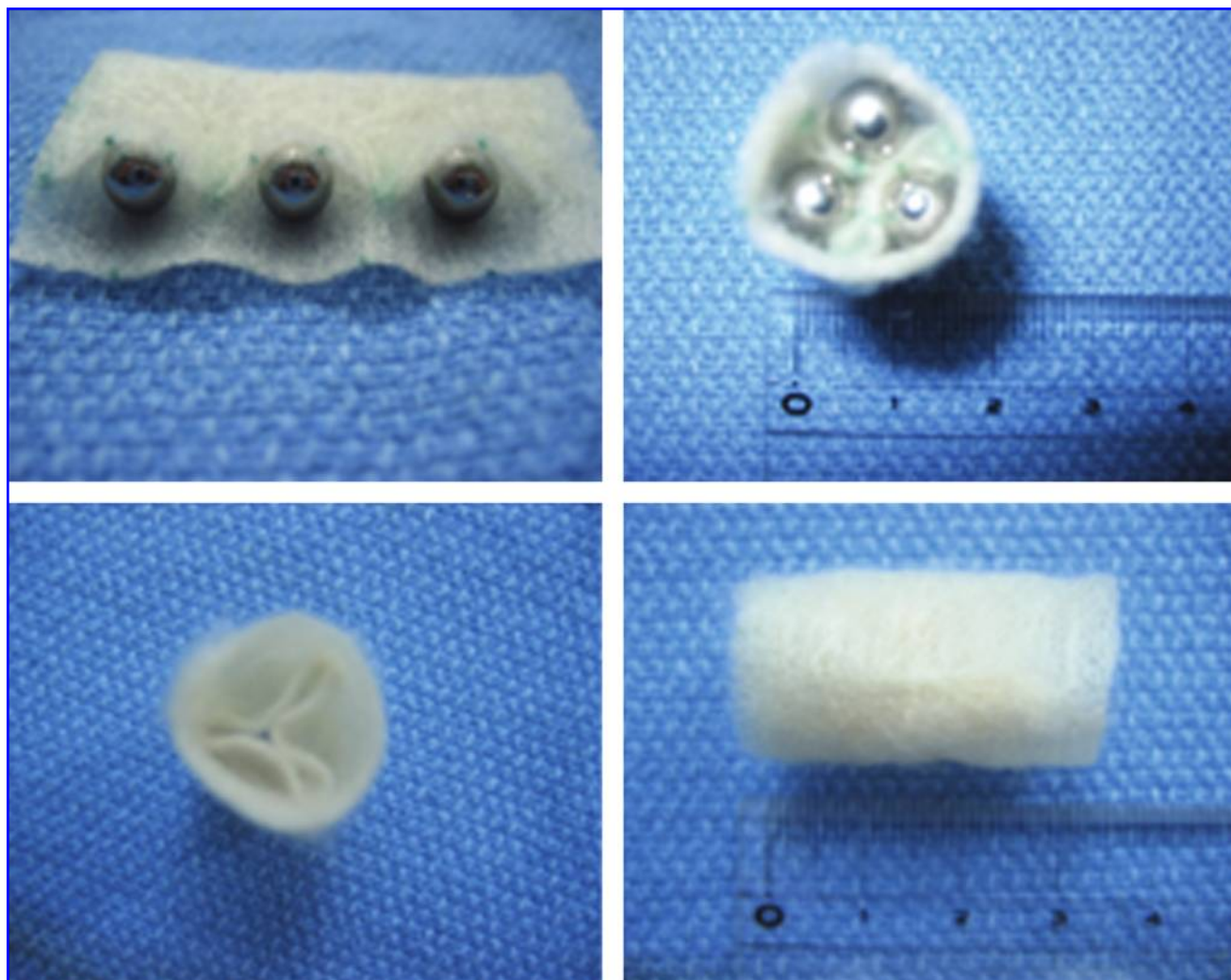

FIG. 2. Properties of PGA/poly-4-hydroxybutyrate scaffold. Schematic illustrating the method of trileaflet valved conduit scaffold fabrication from nonwoven PGA using a needle punching technique. PGA, polyglycolic acid. Color images available online at www.liebertonline.com/ten.
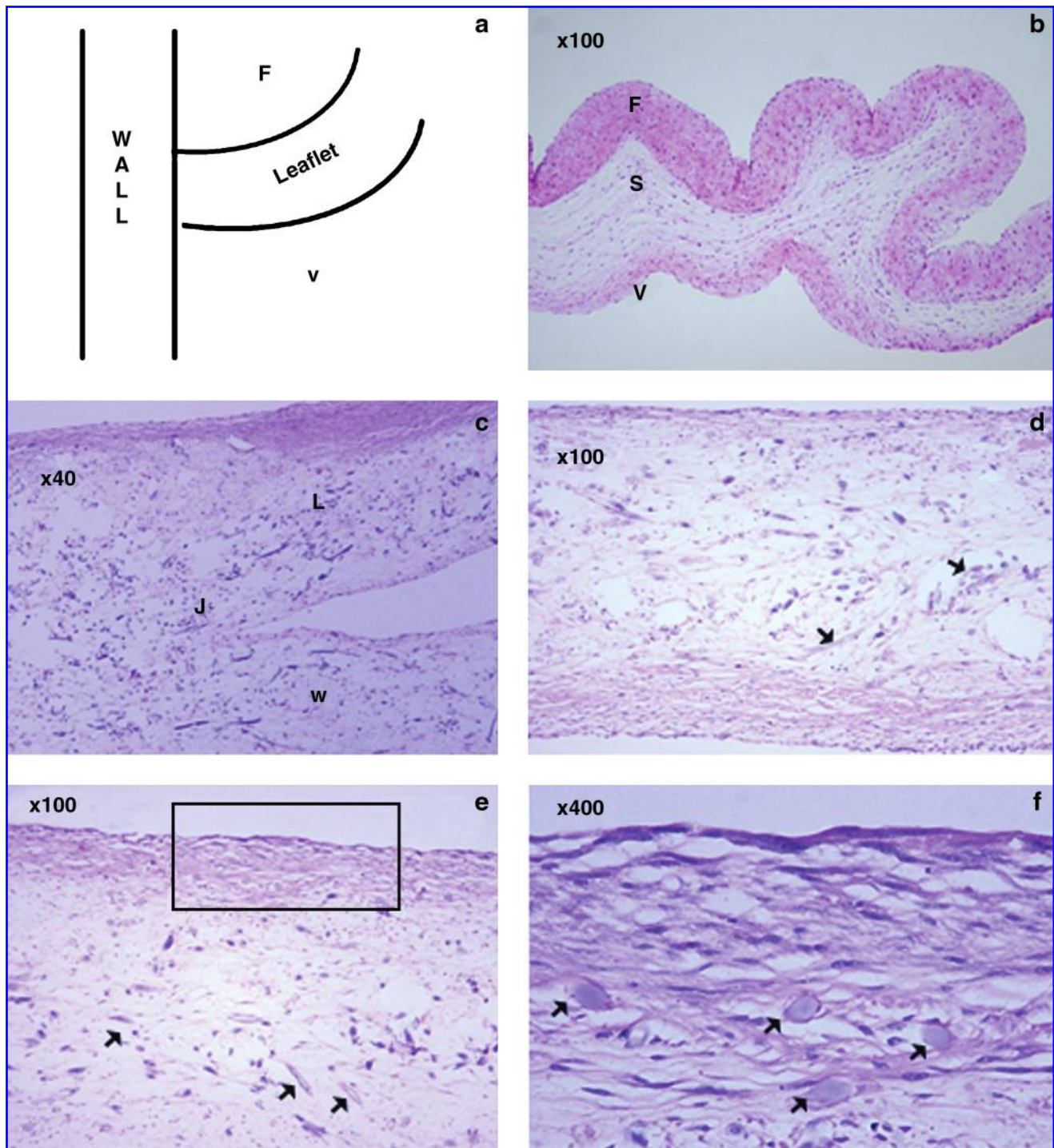
FIG. 3. Tissue formation of EPC-TEHV *in vitro*. (**a**) Schematic of tissue analysis of EPC-TEHV. (**b**) Hematoxylin and eosin staining of native PV and (**c–f**) TE valved conduits. Longitudinal sections of the TE valved conduits demonstrate organized, cellular, and denser tissue formation in the wall and leaflet. (**e**) Luminal surface of the leaflet (Rectangular Box). In (**f**) high-power view demonstrating multilayered and cellular architecture with residual polymers (arrows). Original magnification, ×40, ×100, and ×400. J, junction; L, leaflet; W, wall; F, fibrosa; S, spongiosa; and V, ventricularis. Results were similar in four-valved conduits. TEHV, tissue-engineered heart valve; PV, pulmonary valve; TE, tissue engineering. Color images available online at www.liebertonline.com/ten.
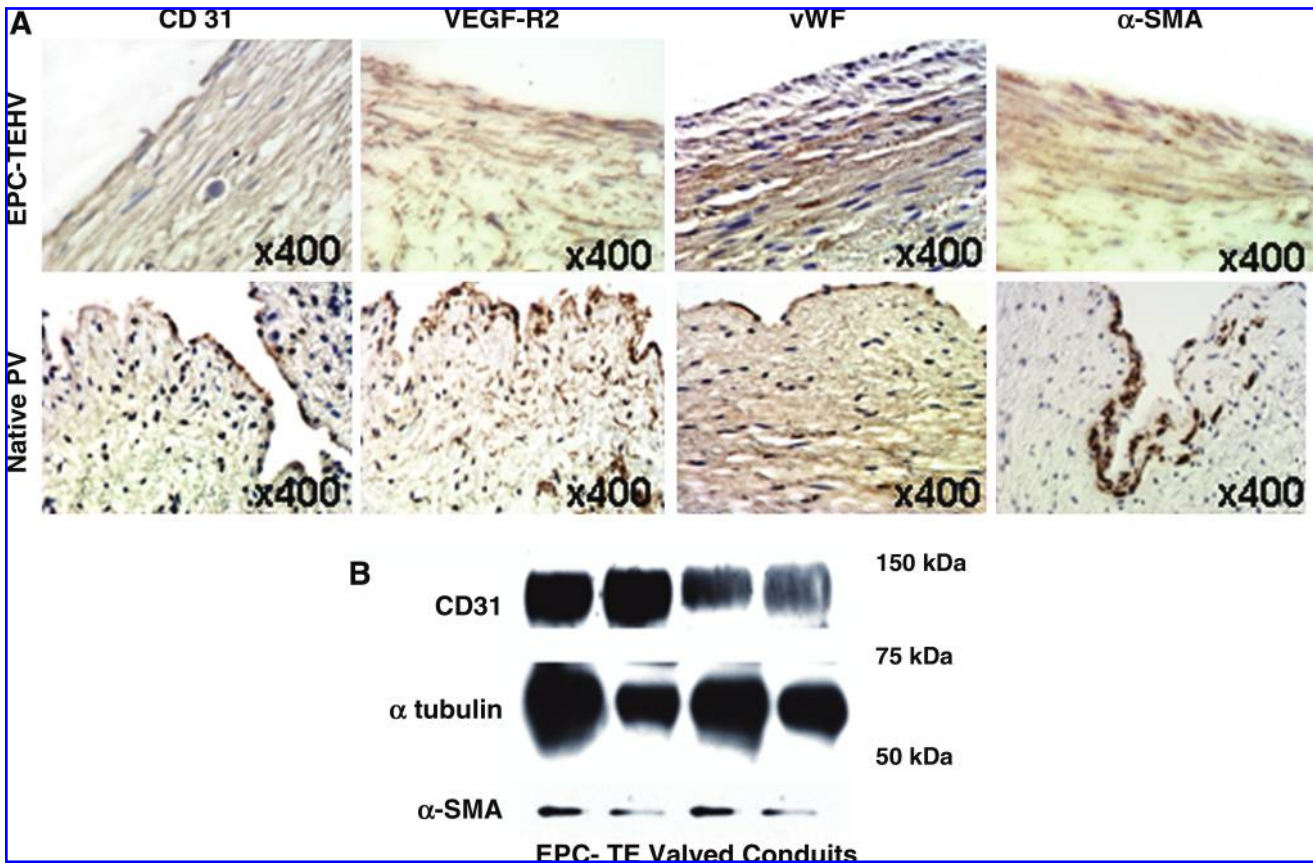
FIG. 4. Expression of EC surface markers and α-SMA in EPC-TEHV *in vitro*. (**A**) High-power views demonstrating expression of CD31 and VEGF-R2 on the luminal surface and α-SMA on the "interstitial" layer of the EPC-TEHV (top), comparable with the native PV (bottom). However, vWF⁺ cells showed expression throughout the constructs (top) with preferential localization in the interstitium, in contrast these cells revealed strong expression pattern in the endothelial layer of the native PV (bottom). Original magnification, ×400. Results were similar in three TE valved conduits. (**B**) Immunoblot revealed expression of CD31 and α-SMA in conduit extracts (10 μg protein). α-Tubulin served as loading control. Representative blot from four different TE valved conduits is shown. The position of the molecular weights (Mw) is indicated (kDa). Color images available online at www.liebertonline.com/ten.

Significant ECM production of EPC-TEHV *in vitro*

Immunostaining of the EPC-TEHV with anti-human LM and FN antibodies revealed ubiquitous expression throughout the seeded scaffold (Fig. 6A). In contrast, EL and Coll I/III are expressed preferentially on the surface and intensely in the interstitium of the EPC-TEHV, respectively. The intensity and localization of ECM expression by the EPC-TEHV were comparable to the native PV.[16,17] Immunoblotting was performed on unseeded scaffolds and on samples of the wall of EPC-TEHV. Expressions of Coll I/III, TE, EL, FN, and LM were observed in seeded scaffold (Fig. 6B). At day 21, protein levels in EPC-TEHV exceeded those in unseeded controls (FN: 3.4, LM: 3.2, Coll III: 1.4, EL: 2.2, $p < 0.0001$; Coll I: 0.9, TE: 2.4, $p < 0.001$) (Fig. 6B). This suggests that EPC themselves are capable of synthesizing their own ECM expression on the scaffold.

EPC-TEHV exhibits dynamic matrix remodeling *in vitro*

After 7, 14, and 21 days, seeded constructs were analyzed by zymography and reverse zymography. MMP-2 is com-

monly found in native PV and was present both in the wall and leaflets of the seeded constructs (Fig. 7A). Interestingly, the expression of latent MMP-2 activity in the seeded conduits changed substantially, increasing through days 7 and 14, and then decreasing through day 21 (Fig. 7A). We have also assessed the levels of TIMP activities in these identical conduit samples and compared these to the native PV. After 21 days, the inhibitory activity of the seeded conduits significantly decreased by 52% and 62% ($p < 0.001$) versus seeded constructs after 7 and 14 days, respectively (Fig. 7B). However, there were no significant differences in the inhibitory activities of the seeded constructs after 7 and 14 days. By day 21, EPC-TEHV conduits have TIMP activity levels comparable to the leaflet and wall of the native PV (12 ± 0.6 and 12 ± 2%), respectively. Both MMP and TIMP activity levels were increased after 14 days, suggesting active remodeling of TE constructs.

Interestingly, after 7 days, gelatinase enzymatic activities of approximately 125 to 140 kDa were detected, suggesting potential expression of MMP-9/neutrophil gelatinase–associated lipocalin activity (MMP-9/NGAL) complexes[18] in seeded constructs.
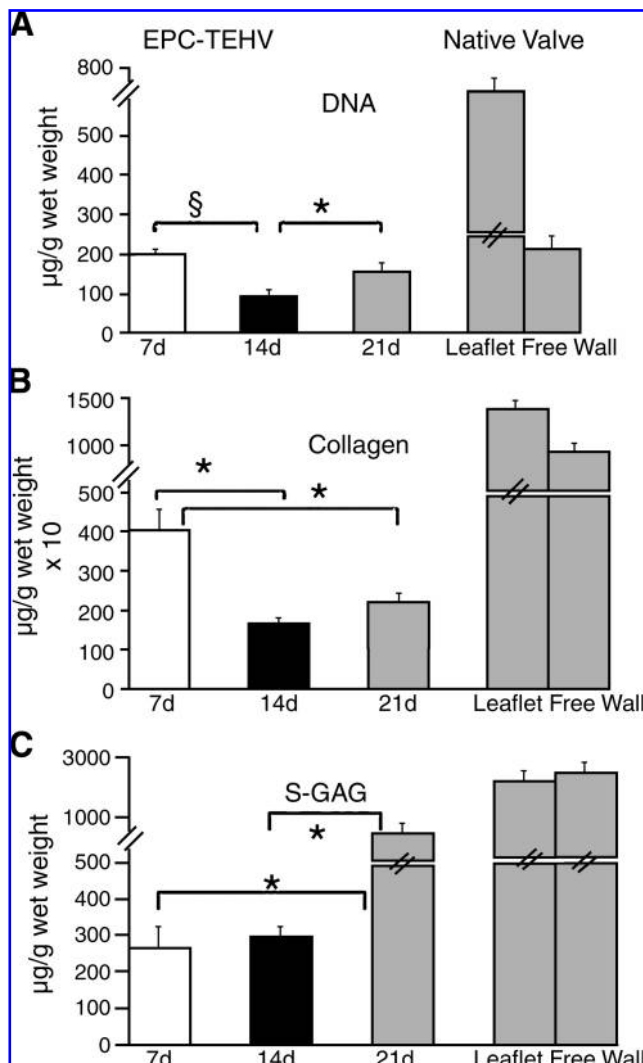
FIG. 5. Effect of cultivation time in EPC-TEHV on DNA, collagen, and S-GAG content. (**A**) DNA, (**B**) collagen, and (**C**) s-GAG content of the seeded constructs after 7, 14, and 21 days. All values were normalized to sample wet weight. Graphic depiction of results from TE constructs ($n = 3$–4 per group) at days 7 revealed increased DNA and collagen by twofold versus days 14 and collagen by 1.8-fold versus days 21 and decreased S-GAG by 2.9-fold versus days 21. Values represent mean ± SEM, $^§p < 0.001$ and $*p < 0.05$. Assays were performed in triplicate. Native PV served as controls. S-GAG, sulfated glycosaminoglycans; SEM, standard error of the mean.

Seeding EPCs increases effective stiffness in PGA/P4HB scaffolds

We examined the mechanical properties of the EPC-TEHV conduits using the three-point bending apparatus (Fig. 8). Following 21 days incubation, the *E* values of the TE valved conduit, as assessed by measurement of the wall, were 7- and 41-fold ($p < 0.001$) greater than that of the unseeded PGA/P4HB scaffold and native ovine PV, respectively.

Discussion

During embryonic valve development, cardiac cushions appear as localized expansions of ECM, known as cardiac jelly. Epicardial and endocardial (endothelial) cells transform into a mesenchymal phenotype, characterized by α-SMA expression, which then invade the underlying cardiac jelly and generate fundamentally different tissues. The process of mesenchymal transformation is a critical initial event in cardiovascular morphogenesis, including heart valve development, coronary artery formation, and inflow and outflow tract septation.[19] Subsequent steps "remodel" fully mesenchymalized cushions into functional, mechanically competent structures (such as septa and valves) and continue throughout human fetal and postnatal development.[20]

CD31+ and α-SMA+ cells are spatially organized in the development of EPC-TEHV

Consistent with the previous studies[3,4,7] we demonstrate in this report that when seeded onto biodegradable valved conduits, subpopulations of EPC-derived cells will transdifferentiate into myofibroblast/SM-like cells closely mimicking cardiac valve interstitial cells.

One of the most important findings in this study is that CD31+ and α-SMA+ cells are spatially organized on the luminal surface and interstitium, respectively, of the seeded scaffold reminiscent of the cellular organization seen in adult cardiac valves. In addition, we speculate that the luminal CD31+ cells are also α-SMA+ cells (Fig. 4). These observations are consistent with a previous report. The presence of dual CD31/α-SMA–positive cells suggests that a subset of valve EC has the potential to differentiate toward a mesenchymal phenotype and migrate into the interstitial region of the leaflet.[6] In addition, our EPC-TEHV expressed mature and functional endothelial cells such as VEGF-R2 and vWF.

EPC-TEHV demonstrates tissue formation and significant ECM production in vitro

Our valved conduit was developed from PGA/P4HB composite. Histological analyses at day 21 demonstrate cellular ingrowth throughout the valved conduits. However, cellular and layered tissue formation is observed in the surface layer of the tissue-engineered scaffold whereas the interstitium layer demonstrates sparse matrix and more residual scaffold material. This finding is perhaps due to the lack of adequate nutrient diffusion during culture. The proliferation of EPC within the pores of the scaffold and secretion of ECM may occlude the pores, prevent penetration of seeded cells, and decrease the supply of nutrients into the interstices of the matrix.[21] To overcome diffusional limitations of oxygen transport from the construct surface into the interior, preconditioning TE constructs with pulsatile fluid flow signals may enhance culture media delivery to the interstices and improve properties of engineered tissues.[22,23]

Initial studies on PGA/P4HB scaffold seeded with arterial-derived cells showed loss of structural integrity with longer periods of time in aqueous tissue culture environment.[23,24] Changes in *E* reflect both scaffold degradation and *de novo* tissue formation. While the EPC-TEHV cultured under static conditions herein demonstrated increased values compared to the native valve, continued remodeling toward more native-like mechanical properties would be expected following implantation and further scaffold degradation, as has been observed previously in functional TEHV based on the PGA/P4HB scaffold.[25] Our group has demonstrated a

FIG. 6. Significant extracellular matrix production in EPC-TEHV *in vitro*. (**A**) Representative illustrations of LM, FN, Coll I/III, and EL expression (brown) at days 21 in EPC-TEHV with residual polymers (arrow) and compared with native PV. Original magnification ×400. (**B**) Representative immunoblots at days 21 show Coll I/III, EL, TE, FN, and LM in cell lysates from unseeded and seeded conduit extracts (10 μg protein; $n = 4$). α-Tubulin served as loading control. Representative blot from either two or four different constructs is shown. (**C**) Extracellular matrix protein band densities were normalized to that of α-tubulin and are expressed relative to the unseeded conduits. Values represent mean ± SEM, **$p < 0.0001$ and $^{§}p < 0.001$. LM, laminin; FN, fibronectin; Coll I/III, collagen types I and III; EL, elastin. Color images available online at www.liebertonline.com/ten.
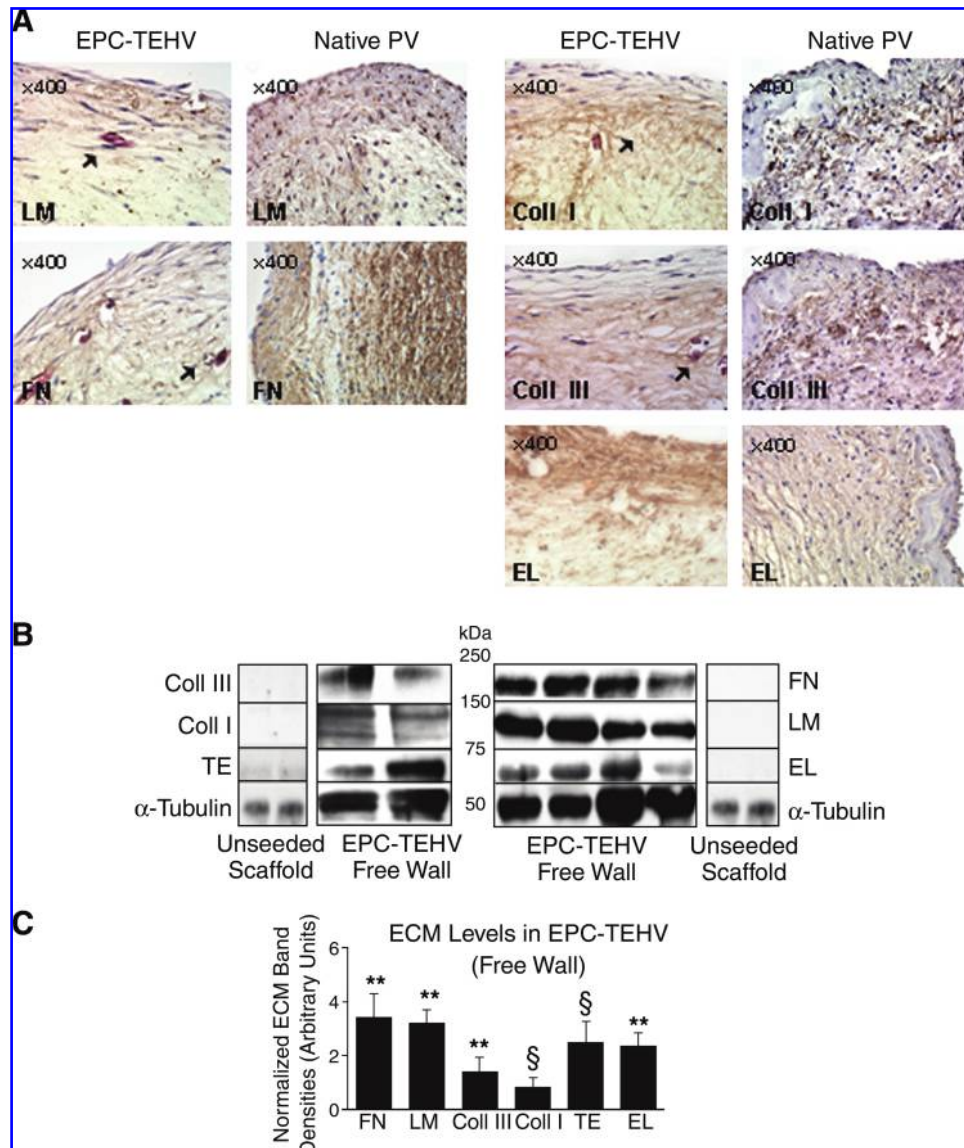
positive linear relationship between the E of the TEHV and its collagen content.[10] Empirically, the collagen secreted by EPC could have also contributed to increase E compared to unseeded scaffold, as well as perhaps to increase the structural–mechanical quality of the PGA/P4HB scaffolding material.[25] As the rate of loss of strength increases, there is an increased requirement for the cells in the TE construct to produce ECM at an earlier time point before implantation.[24] In our current *in vitro* study, after 21 days of seeding and culturing, residual polymer fragments are observed during the time that EPC in the developing tissue are producing appropriate and significant amount of "native valve" matrix proteins (Coll I/III, EL, TE, LM, FN, and GAGs).

EPC-TEHVs are undergoing dynamic ECM remodeling

Previous studies have established a critical role of MMPs, particularly MMP-1, −2, −9, and 13, and their natural inhibitors, TIMPs in the dynamic and adaptive remodeling of adult and, fetal valves, and TEHV.[8,11,26–28] In the present

study, we assessed the potential *in vitro* tissue maturation mechanisms of our EPC-TEHV by studying the pattern of MMPs and TIMPs expression and enzymatic activities at varied incubation times. At day 7, the kinetics of appearance of the MMP-2 and 9, TIMPs, DNA, and ECM components were detected (Figs. 5–7). ECM substantially changed after 14 days with the reduction of ECM and DNA content correlating with decreased levels of MMP-9/NGAL activity but persistent expression of MMP-2 and increased TIMP activity. It has been demonstrated that MMP-9/NGAL complex formation protects MMP-9 from autodegradation, resulting in increased MMP-9 activity.[13,18] These data suggest a shift in the proteolytic equilibrium in favor of MMP inhibition such that MMP activity is suppressed to facilitate tissue formation. The presence of increased TIMP activity levels against a backdrop of decreased MMP activity at day 14 may represent a signal that slows tissue remodeling and ECM formation. Further, the tissue remodeling profile of seeded constructs at day 21 remains constant, where much attenuated and undetectable levels of both MMP-2- and −9, re-
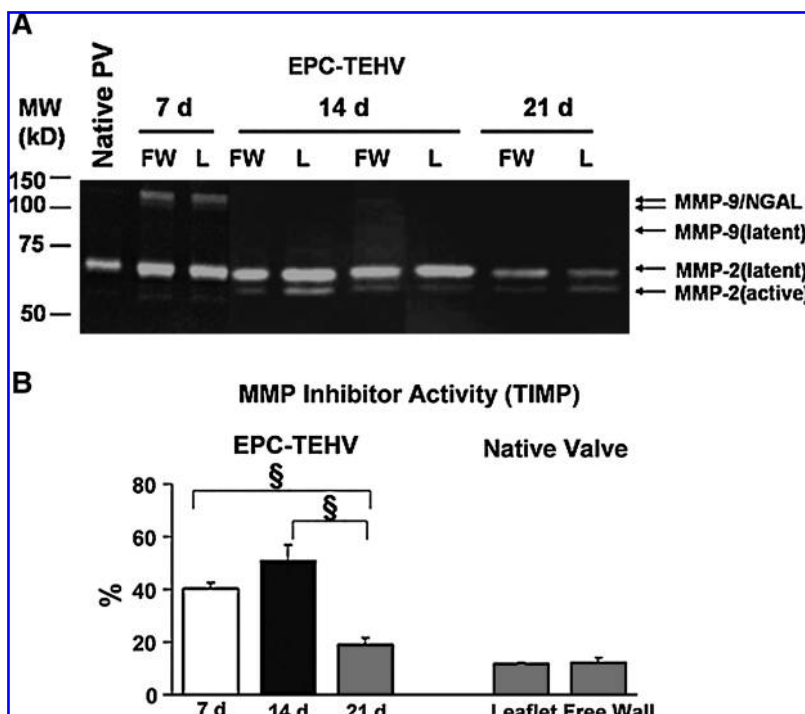
FIG. 7. Temporal expression of MMPs and TIMPs in EPC-TEHV *in vitro*. (**A**) Zymographic analyses of normal valve and TE conduit extracts at different cultivation time points detected significant levels of expression of latent form of MMP-2. Representative zymograms ($n = 2$–4 per group) are shown. (**B**) Levels of TIMP activities were evaluated in the TE valved conduits ($n = 3$–4 per group) after 7, 14, and 21 days. Results from seeded constructs at days 21 reveal statistically decreased TIMP activities by 2- and 2.6-fold versus days 7 and 14, respectively. Assays were performed in duplicate. Values represent mean ± SEM, $^{§}p < 0.001$. Native PV served as controls. L, Leaflet and W, Wall. MMP, matrix metalloproteinase; TIMP, tissue inhibitors of metalloproteinase.

spectively, and low TIMP activity levels were observed. These lower levels of MMPs correlate with increase in DNA and ECM content, compared with day 7, thereby supporting the idea that the TE constructs are undergoing dynamic ECM remodeling. These events occur similarly during postnatal life wherein activated cells gradually become quiescent; MMP–collagenase expression becomes negligible, whereas collagen matures. This suggests a progressive adaptation to the prevailing hemodynamic environment.[28]
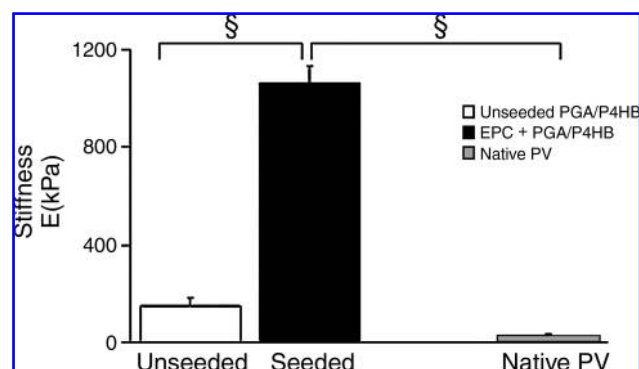

FIG. 8. Effect of seeding EPC on effective stiffness of the PGA/poly-4-hydroxybutyrate samples of the EPC-TEHV conduit wall was tested for *E* using a three-point bending apparatus. Unseeded scaffolds and native ovine PV served as controls. Flexure testing demonstrated significantly increased effective stiffness by 86% and 98% in the EPC-TEHV compared to unseeded scaffolds incubated for 3 weeks in culture medium and native PV, respectively. Values represent mean ± SEM, $^{§}p < 0.001$ versus unseeded. Results from TE constructs ($n = 6$–12 per group).

Study implications

Many of the hallmarks of the initial stage of valvulogenesis are seen in our TEHV, these include expression of α-SMA, ECM production, and MMP-2 expression. For valves to form in the heart, the specific region of endocardial cells lining valve-forming regions undergo a process of activation, migration, and invasion into the cardiac jelly. Although α-SMA is expressed transiently during EMT in developing valves and during steps of endothelial delamination and migration, it is only found in focal regions along the endothelium in healthy postnatal valves.[6,19,28] Mesenchymalized cushion comprises Colls I, II, and III, versican, and other proteoglycans.[29] Migratory mesenchymally transformed endocardial cells secrete MMPs that degrade the endocardial basal lamina, enabling invasion into the cardiac jelly. We demonstrated that our TEHV possess a dynamic/adaptive structure that recapitulates developmental phenotypes of fetal valves, containing cells with an activated/immature phenotype. Our TEHV seems to have undergone a developmental-like event, similar to the EMT valve remodeling process, and this may be used as a template for regenerating living valve alternatives.[20,29] We also demonstrated the potential of EPC to remodel the biodegradable scaffold by expressing ECM components and proteolytic MMPs. These results indicate that cEPCs have the capacity to remodel the three-dimensional scaffold and synthesize matrix, in conjunction with the appropriate environmental (growth factors, cytokines, and ECM) and mechanical cues to guide the development of a TEHV.

Future studies defining the transcriptional regulators that orchestrate the plasticity of progenitor cells in the remodeling events of TEHV to ensure appropriate ECM formation in an organized fashion are clearly necessary. A better

understanding of the contribution of biochemical and bio-mechanical signals to guide the development of a living heart valve structure is necessary for future design criteria of TEHV. Further noninvasive imaging studies in predicting the quality of TEHV constructs and monitoring biological processes during healing and remodeling by observation of MMP activity *in vivo* and developing molecular imaging modalities and validated indicators and markers are warranted.[30]

Conclusions

This study suggests that remodeling of the EPC-seeded TEHV occurred *in vitro* resulting in an organized cellular architecture capable of ECM production. It also demonstrated that cEPC could potentially provide both interstitial and endothelial functions allowing a single-cell source for construction of autologous heart valves.

Acknowledgments

Disclosure Statement

No competing financial interests exist. D.P.M is an employee of Tepha, Inc.

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