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A Novel Single-Step Self-Assembly Approach for the Fabrication of Tissue-Engineered Vascular Constructs

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There is a clinical need for a functional tissue-engineered blood vessel because small-caliber arterial graft (<5 mm) applications are limited by the availability of suitable autologous vessels and suboptimal performances of synthetic grafts. This study presents an analysis of the mechanical properties of tissue-engineered vascular constructs produced using a novel single-step self-assembly approach. Briefly, the tissue-engineered vascular media were produced by culturing smooth muscle cell in the presence of sodium L-ascorbate until the formation of a cohesive tissue sheet. This sheet was then rolled around a tubular support to create a media construct. Alternatively, the tissue-engineered vascular adventitia was produced by rolling a tissue sheet obtained from dermal fibroblasts or saphenous vein fibroblasts. The standard self-assembly approach to obtain the two-layer tissue-engineered vascular constructs comprising both media and adventitia constructs consists of two steps in which tissue-engineered vascular media were first rolled on a tubular support and a tissue-engineered vascular adventitia was then rolled on top of the first layer. This study reports an original alternative method for assembling tissue-engineered vascular constructs comprising both media and an adventitia in a single step by rolling a continuous tissue sheet containing both cell types contiguously. This tissue sheet was produced by growing smooth muscle cells alongside fibroblasts (saphenous vein fibroblasts or dermal fibroblasts) in the same culture dish separated by a spacer, which is removed later in the culture period. The mechanical strength assessed by uniaxial tensile testing, burst pressure measurements, and viscoelastic behavior evaluated by stepwise stress relaxation tests reveals that the new single-step fabrication method significantly improves the mechanical properties of tissue-engineered vascular construct for both ultimate tensile strength and all the viscoelastic moduli.

Introduction

THE DESIGN OF a functional tissue-engineered blood vessel (TEBV) has been a challenge for a number of years.[1,2] Although materials such as expanded polytetrafluoroethylene and Dacron mesh are widely used as blood vessel substitutes in large-diameter vessel applications, the clinical need for a small-caliber arterial graft (<5 mm) has yet to be adequately addressed because of a limited availability of autologous vessels and suboptimal performances of synthetic grafts. Since the pioneering work of Weinberg and Bell,[3] many different approaches for TEBV construction were investigated. The use of biodegradable polymer scaffolds such as poly(glycolic acid) mesh[4,5] and biologically derived hydrogels such as collagen gels seeded with cells have been investigated.[6–8] Acellular decellularized tissues,[9–11] having the potential advantage of being composed of natural extracellular matrix (ECM), are also of great interest for their mechanical properties and biocompatibility, although their thrombogenic and immunogenic properties remain an issue.[12,13] The self-assembly approach, based on the exclusive use of cells and their ability to produce abundant ECM when cultured in the presence of sodium L-ascorbate, demonstrated the possibility to construct highly resistant human blood vessels comprising an adventitia, a contractile media,[14–16] and an intima using a cell-based tissue engineering method.[14]

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With respect to the functional requirements of a tissue-engineered vascular graft, the mechanical properties are of utmost importance.[17] Thus, the mechanical behavior of vascular constructs produced using the self-assembly approach of tissue engineering were investigated in this study. The influences of cell types and production methods on the mechanical properties of these constructs were determined. Three cell types were investigated: vascular smooth muscle cells (SMCs), dermal fibroblasts (DFs), and saphenous vein fibroblasts (SVFs). Previous studies published by our group demonstrated the ability of DFs and SVFs to produce tissue-engineered vascular constructs using the self-assembly approach.[14,18] These cell types were used because they both are potential fibroblast sources for the production of TEBVs for clinical applications. Moreover, two assembly methods were compared: the standard self-assembly and a new single-step approach to produce the engineered tissues. The mechanical properties of self-assembled tissue-engineered vascular constructs were dependent on the cell types used for the construction, because DFs produced stronger tissue than SVFs and SMCs. Each of the vascular constructs studied here presented mechanical properties suggesting a potential suitability for acute mechanical function upon engraftment, based on their associated burst pressures and ultimate tensile strength (UTS). However, improvement was obtained with the new assembly method, allowing the production of tissue-engineered vascular constructs comprising both media and an adventitia (TEVMA) in a single step and facilitating considerably the fabrication of the engineered tissues.

Materials and Methods

This study was approved by the CHA Universitaire de Québec Institutional Review Committee for the Protection of Human Subjects. Tissues were obtained after informed consent was given.

Cell isolation and culture

SMCs were isolated from a human umbilical cord as described previously.[14] Briefly, an umbilical cord was obtained from a healthy newborn and processed immediately. The umbilical vein was rinsed with phosphate-buffered saline (PBS), opened longitudinally, and pinned to a dissection board with the lumen facing upward. Endothelial cells (ECs) were gently scraped from the underlying basement membrane using a scalpel blade without grossly damaging the subendothelial layer. Bands of the thin underlying media layer were then collected, cut into smaller pieces, and placed in a gelatin-coated Petri dish to allow the outgrowth cells to attach to the gelatin-coated dish. Explants were cultured in Dulbecco-Vogt's modified Eagle's medium with Ham's F12 (DMEM-Ham, ratio 3:1; Invitrogen, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT) and antibiotics (penicillin [100 U/mL; Sigma, Oakville, ON, Canada], gentamycin [25 μg/mL; Schering, Pointe-Claire, QC, Canada]), until cells migrated out of the biopsy samples.

SVFs were isolated from a human saphenous vein obtained from an adult volunteer following a surgical vein stripping procedure using the previously described procedure,[18] using the external layer of the vein.

Tissue samples from each cell isolation phase were processed for histological analysis. The results confirmed that explants were harvested from the intended layer of the blood vessel: media for the SMCs and adventitia for the SVFs (data not shown). Two weeks later the cells were trypsinized (0.05% trypsin [Intergen, Toronto, ON, Canada] and 0.01% EDTA [J.T. Baker, Phillipsburg, NY]) and plated at 1×10^4 cells/mm^2 density in noncoated tissue culture flasks (BD Biosciences, Mississauga, ON, Canada). The different cell types were well characterized.[19] Both cell types displayed a constant phenotype during the subculture and immunofluorescent staining of the cells showed that a high percentage of SMCs expressed alpha-SM-actin as well as calponin, whereas only a low number of SVFs expressed these markers.[18,20]

DFs were isolated by human skin biopsy as previously described.[14] Briefly, the dermis was separated from the epidermis by incubation in thermolysine (Sigma). DFs were enzymatically dissociated from the dermis using collagenase H (Roche, Indianapolis, IN), centrifuged, plated in tissue culture flasks, and cultured in DMEM supplemented with 10% FBS and antibiotics.

All cell types were maintained at 37°C in a humidified incubator containing 8% CO_2 and the medium was changed three times per week as described previously.[14] Cells between passages 3 and 7 were used for tissue production.

Tissue-engineered vascular constructs

Tissue-engineered vascular constructs were produced using the tissue engineering method previously described,[14,18] namely the self-assembly approach. Cells were seeded at a density of 1×10^4 cells/cm^2 in gelatin-coated tissue culture flasks and cultured in DMEM-Ham (3:1) supplemented with either 10% FBS in the case of constructs containing DF or 30% FBS and 20 μg/mL EC growth supplement (Calbiochem, San Diego, CA) in the case of constructs containing SVFs. Antibiotics were added to DMEM-Ham in both cases, and sodium L-ascorbate (50 μg/mL; Sigma) was added to the culture medium of every vascular construct to stimulate ECM synthesis. SMCs and fibroblasts were cultured for 14 and 28 days, respectively, until their neosynthesized ECM proteins have self-assembled into an adherent living tissue sheet, which was then gently detached from the culture flask using fine forceps, rolled onto a polystyrene tubular support of 4.5 mm in diameter, and maintained in culture in DMEM-Ham (3:1) supplemented with 10% bovine Fetal Clone II serum (HyClone), antibiotics, and 50 μg/mL sodium ascorbate. Tissue-engineered vascular media (TEVM) were obtained by rolling a SMC tissue sheet, whereas tissue-engineered vascular adventitia (TEVA) were obtained by rolling either a SVF or DF tissue sheet on a tubular support (Fig. 1A).

In the case of TEVMA, two different assembly methods were investigated. The first method used consisted of rolling a sheet of SMCs on a tubular support and superimposing a sheet of DFs on the same support to obtain a TEVMA construct (Fig. 1B). This fabrication method will be referred to as the standard assembly approach (stdTEVMA-DF).[14] The second method is a new technique that allows the fabrication of TEVMA in a single assembly step (ssTEVMA). SMCs and either SVFs or DFs were seeded in two distinct compart-

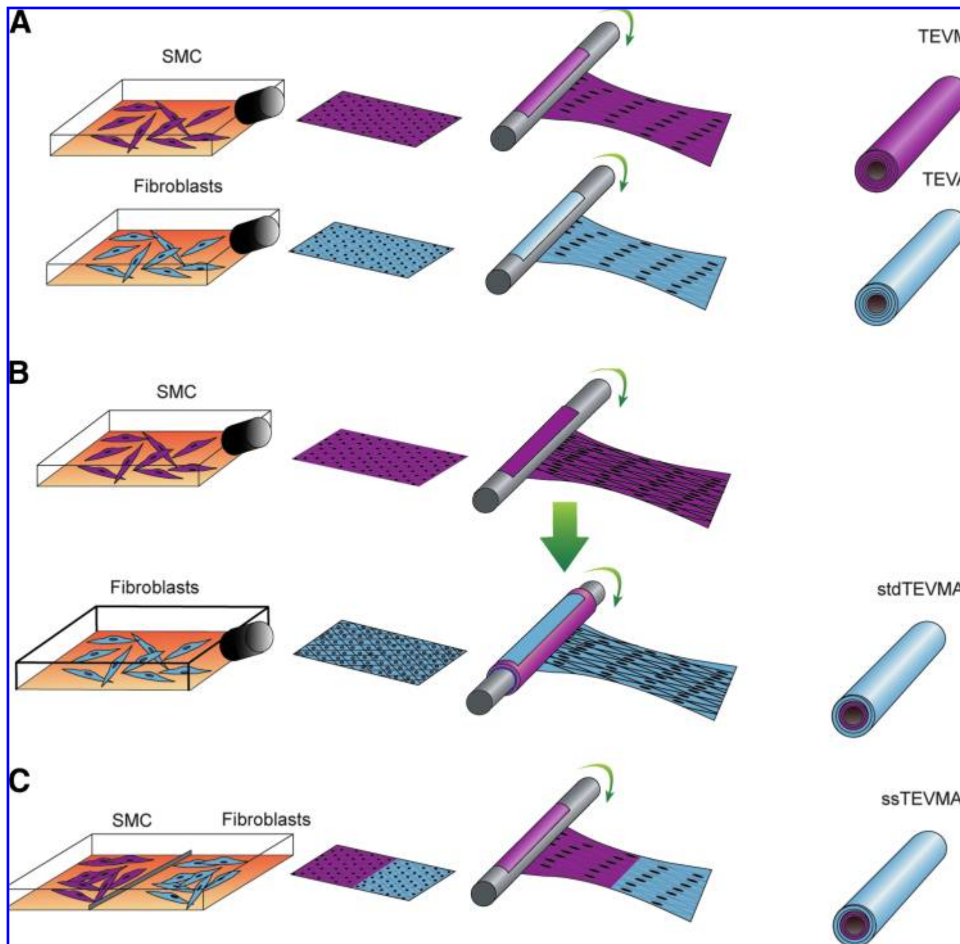

FIG. 1. Schematic view of the different assembly methods used to produce vascular constructs. Tissue-engineered vascular media (TEVM) and tissue-engineered vascular adventitia (TEVA) are assembled by rolling a single cell sheet of either smooth muscle cells (SMCs) or fibroblasts (**A**). The standard tissue-engineered vascular constructs comprising both media and an adventitia (stdTEVMA) assembly requires rolling a fibroblast tissue sheet on top of a SMC tissue sheet (**B**). The single-step TEVMA (ssTEVMA) is produced in a single assembly manipulation because SMCs and fibroblasts are comprised in the same tissue sheet (**C**). Note that the fabrication of both stdTEVMA and ssTEVMA requires the same amount of initial tissue. Color images available online at www.liebertonline.com/ten.

ments of a gelatin-coated tissue culture plate (Corning, Lowell, MA) separated by a custom designed spacer. The spacer was removed at 24 h following cell seeding, to allow cell adhesion to the underlying gelatin-coated tissue culture plate. Then the two cell types migrated toward each other to form a contiguous sheet of tissue containing SMCs and either SVFs (ssTEVMA-SVF) or DFs (ssTEVMA-DF) at the end of the culture period. This tissue sheet was then rolled on a tubular support in a single step, increasing the efficiency of assembly and reducing the amount of operations required for TEVMA production.

All vascular constructs were maintained for a 14-day culture period on the tubular support at 37°C in a humidified incubator containing 8% CO_2. The culture medium was changed three times per week.

Histology and immunodetection

After a 14-day culture period on the tubular support, biopsies of each type of vascular constructs were fixed overnight in Histochoice (Amresco, Solon, OH) and embedded in paraffin. Five-micrometer-thick sections were stained with Masson's trichrome and imaged using a (Nikon, Mississauga, Canada) microscope (Nikon Eclipse TS100). Indirect immunofluorescence detection was performed on frozen sections after fixation in methanol for 10 min at −20°C using a rabbit anti-human elastin antibody (A. Grimaud, Institut Pasteur, Lyon, France) with an Alexa Fluor 594-labeled chicken anti-rabbit immunoglobulin G and a mouse anti-collagen I (Calbiochem) with an Alexa Fluor 594-labeled donkey anti-mouse immunoglobulin G (Sigma). Primary antibody was omitted for controls.

Uniaxial tensile testing

Tissue-engineered vascular constructs were subjected to tensile ring testing[6,21,22] on an Instron 5842 mechanical tester (Instron Corporation, Norwood, MA) after a 14-day culture period on the tubular support. Constructs were cut into 5 mm ring samples and mounted between two hooks adapted to the mechanical tester. The hook-to-hook distance represented the gauge length of the samples. Samples were preconditioned with three cyclic loading sequences estimated to 20% of failure strain prior to testing (data not shown). The rings were loaded to failure at a displacement rate of 0.2 mm/s. UTS and failure strain were defined by the peak stress and maximum deformation withstood by the samples prior to failure. Linear modulus[22] was defined as the slope of the linear portion of the stress–strain curve comprised between 25% and 80% of the UTS of the sample. Note that engineering stresses were calculated by dividing the recorded loads by the cross-sectional area of the sample using initial construct dimensions, and engineering strain was used to measure the deformation of the vascular constructs. Stress–strain curves were plotted and analyzed using an in-house–developed Matlab® (The Mathworks, Natick,

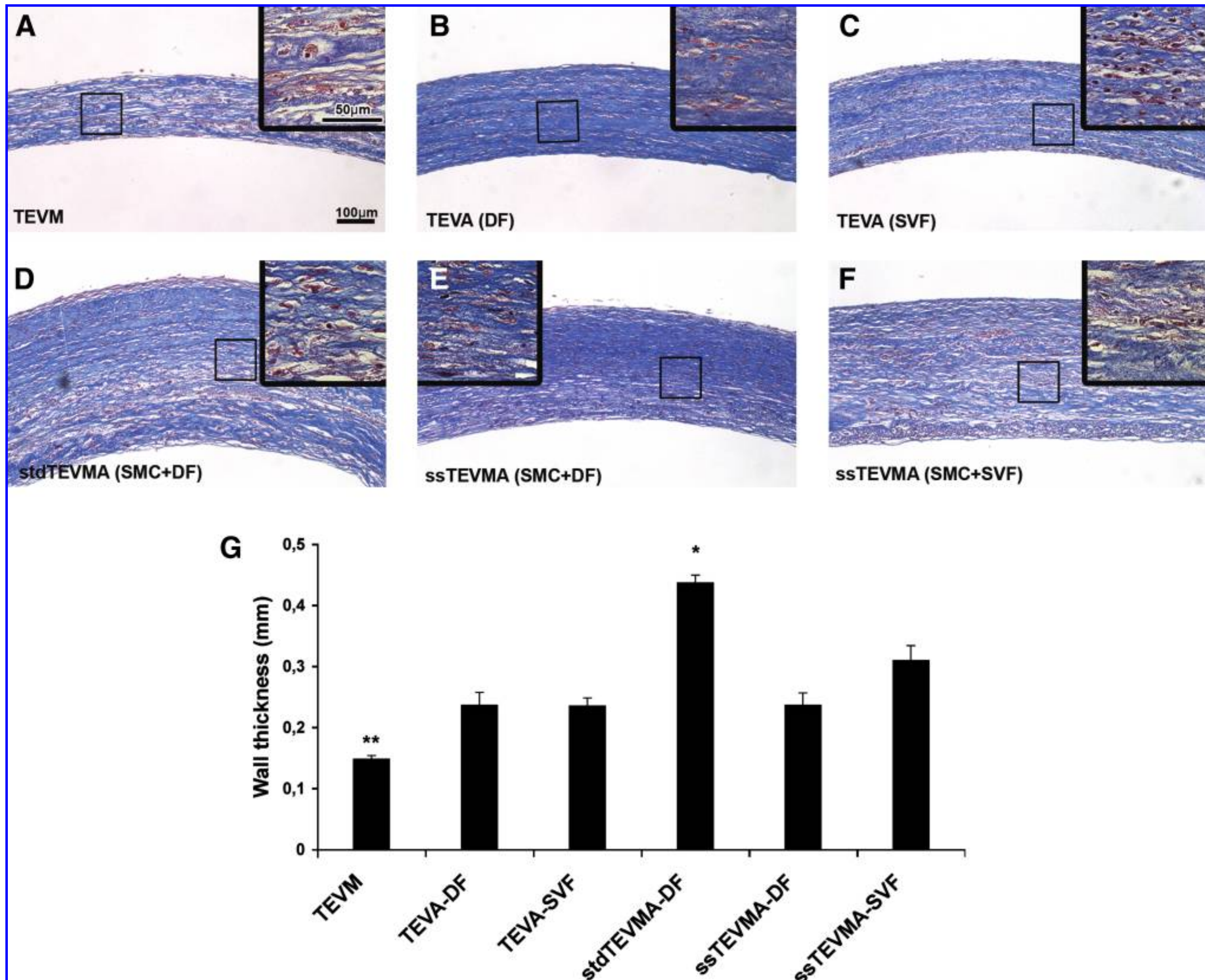

FIG. 2. Histological cross sections stained with Masson's trichrome (**A–F**) and wall thickness (**G**) of the different tissue-engineered vascular constructs. Higher magnifications of the vascular constructs (black boxes) clearly demonstrate that extracellular matrix density and tissue thickness are both dependant on cell type and assembly method. *Significantly higher wall thickness for the TEVMA assembled using the standard self-assembly method. **Significantly lower wall thickness for the TEVM reconstructed with SMCs when compared with the other vascular constructs.

MA) script to facilitate the calculation of all the tensile testing parameters.

Burst pressure testing

Burst pressure measurements were performed by inflating the tissue-engineered constructs up to failure using a custom-built experimental setup (Levesque *et al.*, in preparation). Tissues were mounted and secured with o-rings on in-house–designed canulas and were loaded in a chamber containing PBS at 37°C. Vascular constructs were pressurized with PBS using a syringe pump activated by a stepper motor (Excitron, Boulder, CO) controlled by a LabView virtual instrument (National Instruments, Austin, TX) at a constant 4 mL/min flow rate. Pressure data were recorded by a pressure transducer (68846-series; Cole Parmer, Montreal, QC, Canada) connected to an acquisition card (NI PCI-6221; National Instruments) and acquired using the previously described virtual instrument. Burst pressure was considered to be the highest pressure value recorded prior to failure of the construct.

Stepwise stress-relaxation testing

Stress-relaxation testing of the different vascular constructs was performed to assess the viscoelastic behavior of our engineered tissues.[22] Constructs were cut into 5 mm ring samples and mounted between two hooks adapted to the Instron 5842 mechanical tester stated previously. As these tests required an extended period of time, a chamber was designed to allow for the tissue sample to remain in culture media at 37°C during the whole process. Five incremental displacement steps of 10% strain were applied between hold periods of 15, 30, 45, 60, and 60 min, respectively, to allow the tissue to reach equilibrium. The timepoints were chosen on the basis of results demonstrating that peak and equilib-

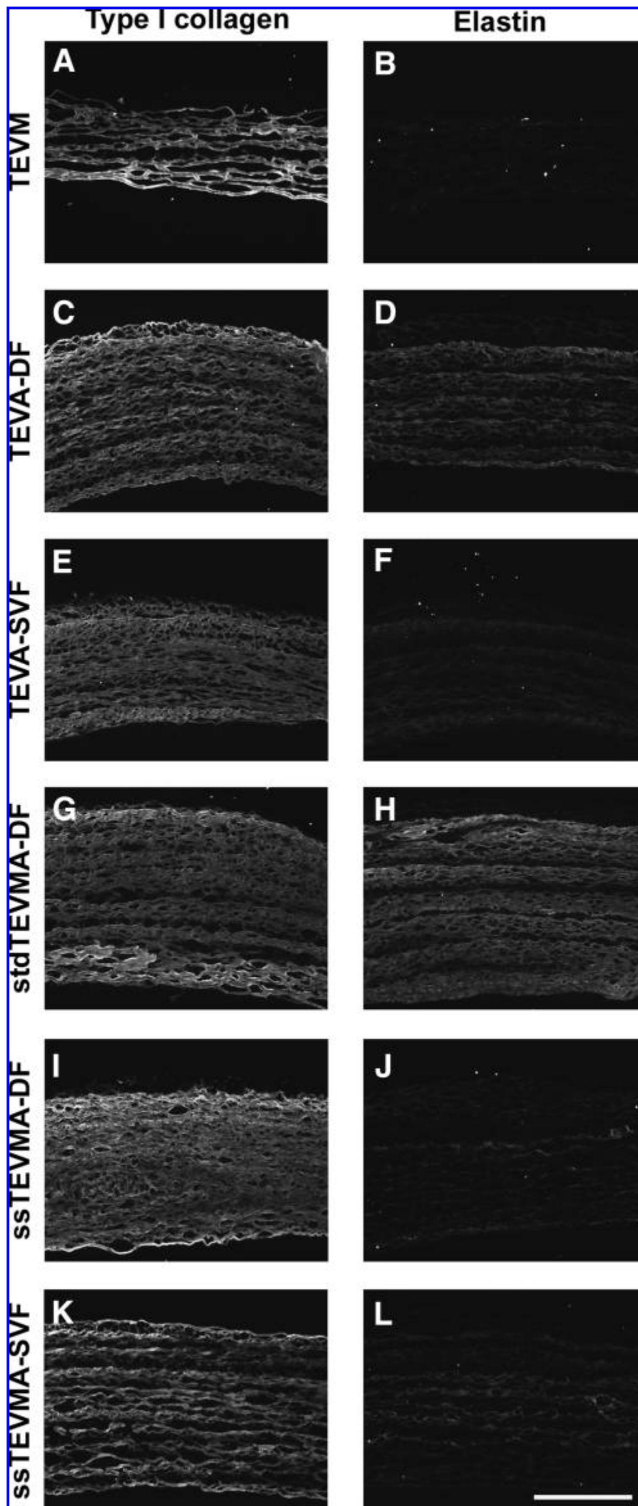

FIG. 3. Characterization of the extracellular matrix by immunofluorescence staining of type I collagen (**A, C, E, G, I, K**) and elastin (**B, D, F, H, J, L**) of tissue-engineered vascular constructs. Collagen I expression is found in all vascular constructs, whereas elastin labeling is present in TEVA and stdTEVMA produced using dermal fibroblasts (DFs) and slightly labeled in ssTEVMA. Scale bar = 200 μm.

rium values measured for each incremental step using these specific parameters were following a linear evolution as a function of time, allowing for a direct measurement of the initial and equilibrium moduli on the stress-relaxation graph (data not shown). Note that the initial modulus is the slope of the best fit of stress as a function of strain immediately following each step, whereas equilibrium modulus is the slope of the best fit of stress as a function of strain following each relaxation period. Stress-relaxation data were plotted and analyzed using an in-house–developed and carefully validated Matlab script allowing the detection of peak and equilibrium values as well as calculation of the peak and equilibrium moduli.

Statistical analysis

The complete experiment was repeated two times and results are expressed as mean ± standard error of mean. In the case of uniaxial ring testing and stepwise stress-relaxation testing, a minimum of three distinct sections of the same construct were tested from three different specimens for each condition ($n = 3$ vascular constructs × 6 construct formulations = 18 vascular constructs per experiment) for both tests. For burst pressure, three different specimens of each condition were tested ($n = 3$ vascular constructs × 6 construct formulations = 18 vascular constructs per experiment). Comparison of specific parameters between the different types of constructs was performed using an analysis of variance general linear model and a *post hoc* Tukey test. Data were analyzed using Minitab® (Minitab, State College, PA). Statistical significance was established using a standard p-value of < 0.05.

Results

Histology and immunodetection

The effect of cell types and assembly methods on the histology and tissue thickness observed using Masson's trichrome and immunofluorescence for each vascular construct showed a denser and more compact ECM for TEVA produced using DFs in comparison to SVFs and TEVM constructed with SMCs (Figs. 2A–C and 3A, C, E). TEVM also had a significantly lower wall thickness than TEVA fabricated with either DFs or SVFs (Fig. 2G), suggesting that SMCs produced less ECM than fibroblasts in presence of sodium L-ascorbate. Based on histological analysis and wall thickness, the new assembly method consisting of the fabrication of a ssTEVMA (Fig. 2E) in a single step led to the formation of a more compact ECM when compared with the standard self-assembly approach stdTEVMA (Fig. 2D). Immunofluorescence imaging showed that collagen I is expressed in all vascular constructs with small variations in intensity between the different cell types and assembly methods (Fig. 3). Elastin was found to be present in vascular constructs containing DFs (Fig. 3D, H, J), a small labeling was noticeable in SVF (Fig. 3F, L), whereas the media portion containing SMCs were not labeled (Fig. 3B).

Mechanical strength

Uniaxial tensile testing of all the vascular constructs showed a characteristic stress–strain profile displaying a toe region, followed by a linear region and a rupture point

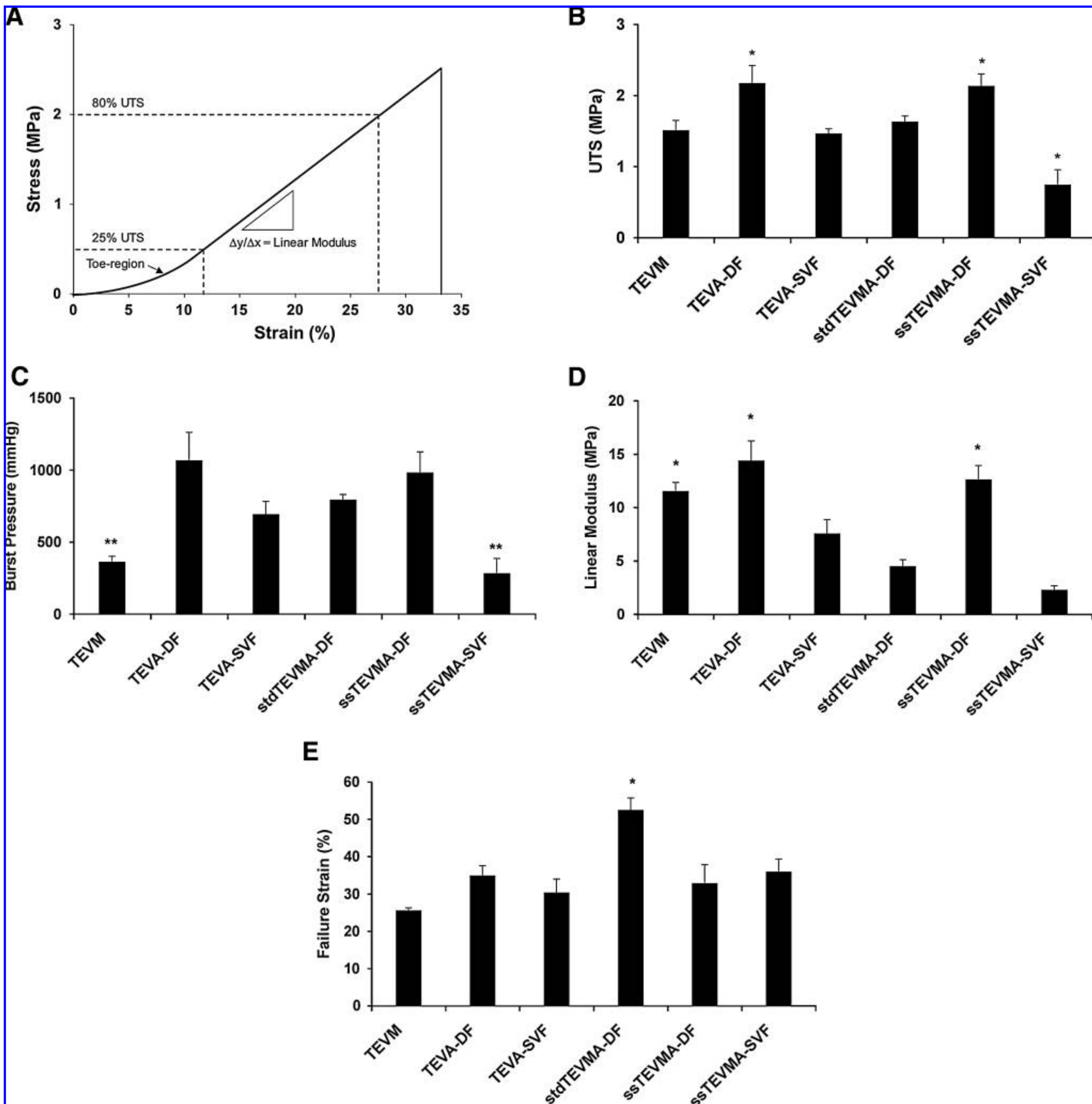

FIG. 4. Influence of the cell type and assembly method on the mechanical properties of the different tissue-engineered vascular constructs. Characteristic stress–strain curve displaying a toe region, followed by a linear region and a rupturing point, defining the ultimate tensile strength (UTS) and failure strain of the engineered tissue (**A**). UTS (**B**) and linear modulus (**D**) results indicate that vascular constructs produced using DFs have superior mechanical strength in comparison to those produced with saphenous vein fibroblasts (SVFs). These data, when compared with burst pressure results (**C**), show that our new assembly method increases the mechanical strength of our ssTEVMA-DF. Failure strain data (**E**) demonstrate that all the engineered vascular tissues tested are able to deform over 25% and that the standard self-assembly method allows the stdTEVMA to deform significantly more than the other vascular constructs. Note that every vascular construct tested had a characteristic stress–strain profile displaying a toe region, followed by a linear region and a rupturing point (data not shown). *Superior statistical significance in comparison to other conditions. **Inferior statistical significance in comparison to other conditions.

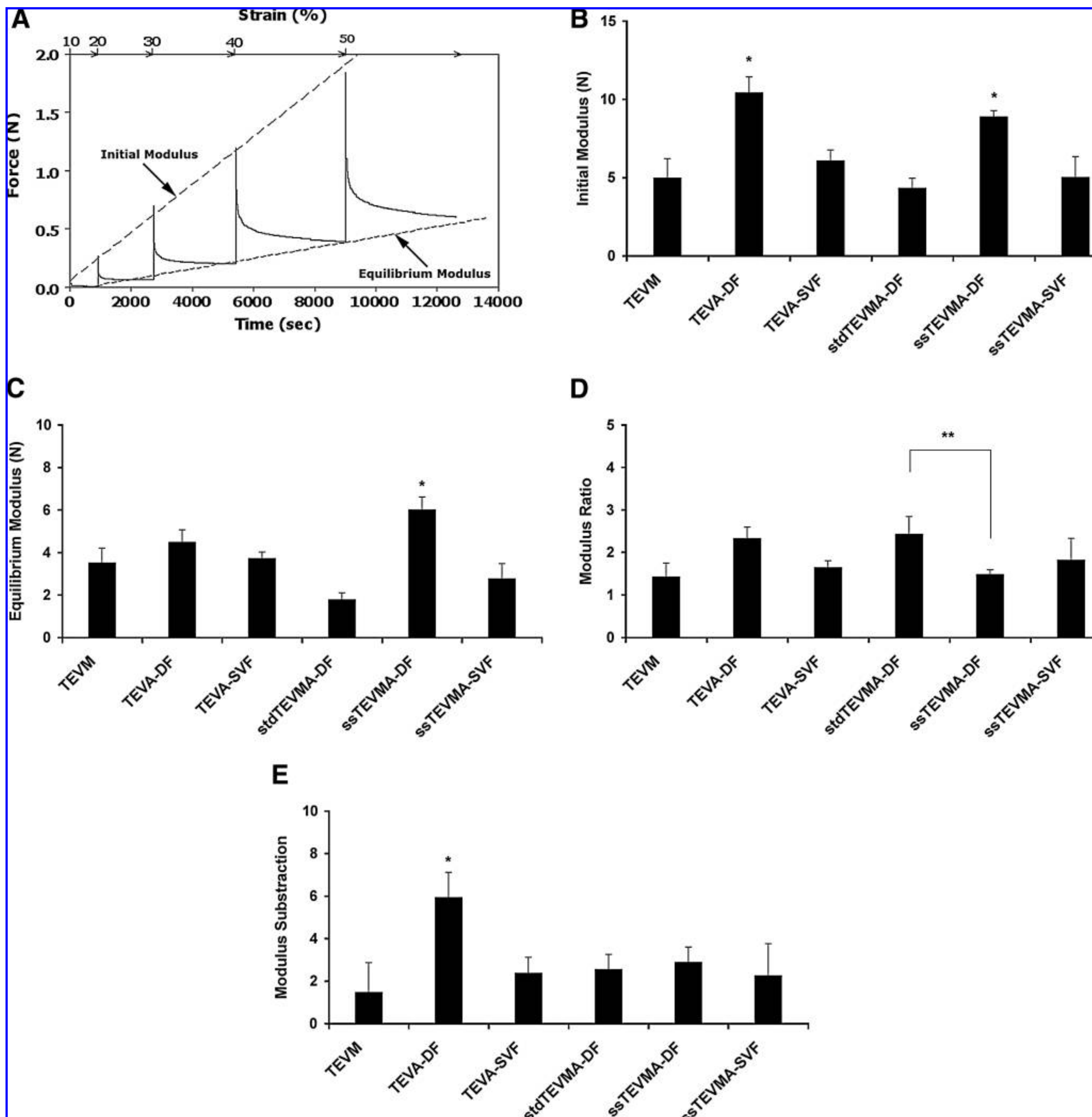

FIG. 5. Stepwise stress-relaxation data obtained for the different vascular constructs. Characteristic time-dependent stress-relaxation profile (**A**) showing the initial modulus (top large dashed line) and equilibrium modulus (bottom fine dashed line). Initial modulus data (**B**) showing that TEVA reconstructed using DF and TEVMA combining DF and the new single-step assembly method are able to bear higher loads following a step deformation. This observation can also be applied in terms of equilibrium modulus (**C**), meaning that the loads at which these constructs are reaching equilibrium are superior to those of the other vascular constructs. Modulus ratio (**D**) indicating whether vascular constructs' viscoelastic behavior is dominated by viscous or elastic attributes. Modulus substraction (**E**) allowing for comparison of the viscous and elastic components between the different vascular constructs. *Superior statistical significance. **Statistical significance between two conditions.

typical of biological tissues (Fig. 4A).[23] UTS measurements (Fig. 4B) showed that TEVA engineered with DFs displayed an increase when compared with TEVA-SVF or TEVM. The same observations can be made from the burst pressure measurements (Fig. 4C). When compared with the stdTEVMA-DF assembly, the ssTEVMA-DF yielded significantly higher UTS and increased burst pressure results (Fig. 4C), indicating an improvement for test to failure. As anticipated, results for UTS and burst pressure were significantly lower for ssTEVMA constructed with SVFs compared with DFs (Fig. 4B, C), correlating with the differences obtained for TEVA produced with these two types of fibroblasts. SMCs

and DFs produced a stiffer tissue than SVFs as shown on linear modulus data (Fig. 4D). The ssTEVMA-DF assembled using the new method had a linear modulus in the same range than that of TEVM and TEVA produced with DFs but higher than TEVMA produced with the standard two-step assembly. This result suggested that the assembly between the media and the adventitia was improved by the new single-step method because the stiffness of individual tissues was reproduced in these ssTEVMA-DF in contrast to TEVMA produced with the standard self-assembly method using DFs. All vascular constructs were able to sustain strains up to 25% without failure, with the maximum strain prior to failure being withstood by the stdTEVMA-DF (~ 50%) as shown in the failure strain results (Fig. 4E). This result correlates with the histology report showing a tissue experiencing less compaction for the stdTEVMA-DF when compared with the ssTEVMA-DF, therefore suggesting an increased fluid flow within the structure and an easier displacement of the ECM components of the tissue during the tensile ring testing, resulting in an increase in failure strain for this condition.

Viscoelastic properties

Stress-relaxation profiles obtained for the vascular constructs were characterized by a peak value, followed by an exponential decay of the stress measured in the tissue, because of fluid flow and deformation of the ECM components with time (Fig. 5A). Stress-relaxation curves demonstrated that vascular tissues engineered using the self-assembly were all able to sustain a load for an extended period of time (data not shown). The initial and equilibrium moduli (Fig. 5B, C) were evaluated to compare the stress-relaxation behavior of the different vascular constructs. Initial modulus was significantly higher for both TEVA and ssTEVMA produced using DF, whereas stdTEVMA-DF and constructs produced using SVFs all had lower initial moduli (Fig. 5B). This translated into the capacity of the TEVA and the ssTEVMA produced using DFs to bear a higher load for a certain strain level than the other constructs. The equilibrium modulus was calculated to evaluate the ability of a tissue to withstand a given load for an extended period of time. ssTEVMA-DF fabricated by the new self-assembly approach had a significantly higher equilibrium modulus than its counterparts (Fig. 5C). This could also be attributed to the fact that a potentially improved cohesion between the media and the adventitia is achieved with this single-step assembly method, maximizing the contribution of both layers to bear the applied load. The modulus ratio (Fig. 5D) was calculated to determine whether these tissues were dominated by viscous or elastic attributes. In the case of our tissue-engineered vascular constructs, all the modulus ratios ranged between 1.3 (ssTEVMA) and 2.5 (stdTEVMA), indicating that all the tissues tested were displaying a strong elastic behavior (Fig. 5D). The modulus subtraction (Fig. 5E) represents the difference between the initial modulus and the equilibrium modulus calculated for every type of construct. This parameter indicates whether the differences between viscoelasticity of different tissues are due to viscous or elastic components. All the vascular constructs displayed a modulus subtraction in the same order of magnitude, showing that although they have different elastic components, the viscous behavior of the engineered-tissue remains essentially the same, except for the TEVA-DF, which displays a significantly higher modulus subtraction, indicating a more viscous behavior when compared with the other constructs. These results indicate that the new single-step assembly method increases the elastic attribute of the ssTEVMA-DF when compared with stdTEVMA-DF and that the fluid-like behavior of these tissues remains the same, both parameters being essential for the mechanical function of a TEBV.

Discussion

This report highlights a new fabrication method of assembling TEVMA using a single-step process (ssTEVMA) that enhanced the UTS, linear modulus, and modulus ratio of living vascular constructs produced *in vitro* using DFs, when compared with the standard self-assembly approach. From an engineering standpoint, the single-step assembly technique has a tremendous advantage when compared with the standard assembly method. The fact that only one single rolling operation is required is very important because this step requires an individual to manipulate the tissue. Therefore, reducing from two steps to only one for the rolling part of the assembly is crucial when trying to develop scalable tissue engineering methods because it reduces the risk of contamination and greatly improves the ease of assembly of the TEVMA. Further, it showed the capacity of the self-assembly approach to allow the fabrication of vascular constructs with significant mechanical properties independently of the cell type used. Mechanical properties of tissue-engineered vascular constructs produced by the self-assembly approach rely on the ECM produced by SMCs and fibroblasts. The aim of this study was to demonstrate the efficiency of the new single-step assembly method and the resulting mechanical properties. Although essential to produce a functional TEBV, ECs were not included in the vascular constructs under the assumption that they do not contribute significantly to the mechanical properties of blood vessels.[24] However, the contribution of the ECs to the mechanical behavior of TEBVs should not be overlooked because these cells are known to secrete biochemical factors such as transforming growth factor-beta,[25] which is known to influence tissue homeostasis and regulate ECM production and degradation in engineered tissues.[26–28] Therefore, further studies including ECs in this model will allow us to evaluate the full functionality of this new assembly method.

Histological and immunofluorescence observations obtained correlated with mechanical testing data. Indeed, TEVA and TEVMA produced with DFs had denser and more compact ECM, which resulted in higher mechanical strength and burst pressure when compared with the other cell type. As previously reported by many groups, uniaxial tensile testing performed on ring sections of vascular constructs is one usual method to evaluate the mechanical properties of TEBV.[6,29–31] The significantly higher UTS, linear modulus, and burst pressure of TEVA and ssTEVMA produced with DFs compared with SVFs suggest that fibroblasts isolated from dermis and saphenous vein present different characteristics for tissue engineering. This could be explained by the presence of different levels of protein expression in the ECM as demonstrated in Figure 3. These results are consistent with previous studies showing different biological

properties for fibroblasts from different anatomical locations.[32] This tissue-specific cellular behavior could be an important vascular tissue engineering parameter to account for, because physiological compliance match and elastic response are essential to construct a functional blood vessel.[33]

UTS and burst pressure data of TEVMA produced using DFs with the new assembly method are higher than those engineered using the standard approach, but all constructs presented a UTS within the same order of magnitude as a bovine native artery.[34] These results are consistent with the histology showing the ssTEVMA-DF as a compact tissue where the media and the adventitia are better integrated when compared with stdTEVMA-DF. The match in the linear modulus of TEVM, TEVA-DF, and ssTEVMA-DF also suggests a better integration between the layers because the stress–strain behavior of all three vascular constructs were in the same order of magnitude. Another important observation is that the comparison of UTS and burst pressure results followed the exact same trend for every engineered tissue when compared with each other. This clearly demonstrates that the self-assembly method allows for the production of vascular tissues having uniform mechanical properties throughout their entire length because UTS is a measurement providing strength data on the biological material itself, whereas burst pressure assesses the structural integrity of the tissue as a vascular construct. As the mechanical properties of the different layers (TEVM and TEVA) are not additive when compared with TEVMA, one could question the use of the TEVM constructed using SMCs in the process. Although TEVM do not display mechanical properties as strong as that of TEVA, SMCs comprised in the TEVMA are essential for the functionality of a TEBV when it comes to vasoactivity.[35] Interestingly, the viscoelasticity measurements for ssTEVMA suggest interactions between the cell types, leading to a composite material behaving differently than TEVA alone. Such interactions may also explain the different vasocontractile responses of TEVA, TEVM, and TEVMA in presence of vasoactive agents.[18]

Viscoelastic behavior of the tissue-engineered vascular constructs were evaluated using stepwise stress-relaxation testing similar to those published by Berglund *et al*.[21,22] Although the collected data displayed similar curves, the time required to reach equilibrium following each strain increment was significantly longer for our tissues because of a much stiffer material resulting in a longer stress response as a function of time. Initial and equilibrium moduli indicate whether a construct reaches higher peak values and equilibrates while bearing a higher load. In both cases, ssTEVMA-DF displayed better results, translating to a modulus ratio closer to 1. This result demonstrated that the new method, allowing for the assembly of a TEVMA in a single step, increased the amount of elastic component in response to stress-relaxation testing. Informations regarding the viscoelastic behavior of the vascular constructs are implemented when looking at the modulus subtraction showing the difference between the peak and equilibrium modulus. One can see that although the elastic component is increased for the ssTEVMA when compared with the stdTEVMA, modulus subtraction indicates that the viscous component of both stdTEVMA and ssTEVMA remains unchanged during stress-relaxation testing. Modulus ratio results show that all our constructs displayed elastic attributes in response to stress-relaxation testing, indicating their capacity to fulfill an essential functional requirement for a TEBV.

Previous studies published by our group showed the ability to produce functional vascular constructs using the self-assembly approach.[14,18,36] These results demonstrated the ability of the self-assembly approach to allow the cells to produce their own ECM during the process, to preserve cell morphology throughout the culture, and to improve their specific differentiation characteristics. This study demonstrates that these vascular constructs are able to sustain supraphysiological mechanical stresses and possess better mechanical properties than most TEBVs produced using collagen scaffolds and hydrogels.[29,30] Although current gold standards such as saphenous veins can sustain burst pressures over 1600 mmHg,[37] results showed that the new single-step assembly increases the mechanical strength of self-assembled vascular constructs when compared with the standard approach. We are currently investigating new strategies to increase the mechanical strength and reduce the culture period of our vessels. As we and others previously demonstrated, mechanical stimulation can induce changes in an engineered tissue.[38,39] Based on the work of many investigators,[6,30,34,40,41] we are currently evaluating the capacity of our constructs to react to mechanical stimuli such as cyclic strain, shear stress, and pulsatile flow using bioreactor technology, to improve their functionality in terms of elasticity and physiologic compliance match. Moreover, a new approach that we developed for aligning the consecutive ECM and cell layer using microstructured material resulted in a better physiological organization of the media[32] and may also improve the functionality of our TEBV.

Conclusion

We conclude that our new method for producing tissue-engineered vascular constructs is relevant because it allows for the production of TEVMA in a single assembly step, including both SMCs and DFs, and enhances the burst pressure, UTS, and linear modulus of the ensuing constructs when compared with the standard assembly approach. The use of SMCs and DFs in the production of TEBV is suitable because both cell types are contributing to the mechanical properties as well as to the structural and physiological functionality of engineered tissues. The results have shown that all tissue-engineered vascular constructs produced by the self-assembly approach have the ability to sustain physiological pressures and are displaying viscoelastic behaviors characterized by appropriate elastic attributes. Future work involving the use of this cell-based human *in vitro* model could potentially help to study the influence of physical forces on vascular cells and tissues. Indeed, this model has the capacity to discriminate or associate different blood vessel components, resulting in very strong and precise analysis of fundamental phenomenon. Thus, further investigations of these constructs cultured in bioreactors will allow us to have a better understanding of the influence of physiological and mechanical stimuli on the mechanical behavior of each of these TEBV components.

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Disclosure Statement

No competing financial interests exist.

References

1. Conte, M.S. The ideal small arterial substitute: a search for the Holy Grail? FASEB J **12,** 43, 1998.
2. Nerem, R.M., and Ensley, A.E. The tissue engineering of blood vessels and the heart. Am J Transplant **4 Suppl 6,** 36, 2004.
3. Weinberg, C.B., and Bell, E. A blood vessel model constructed from collagen and cultured vascular cells. Science **231,** 397, 1986.
4. Niklason, L.E., Gao, J., Abbot, W.M., Hirschi, K.K., Houser, S., Marini, R., and Langer, R. Functional arteries grown *in vitro*. Science **284,** 489, 1999.
5. Kim, B.S., Putnam, A.J., Kulik, T.J., and Mooney, D.J. Optimizing seeding and culture methods to engineer smooth muscle tissue on biodegradable polymer matrices. Biotechnol Bioeng **57,** 46, 1998.
6. Seliktar, D., Black, R.A., Vito, R.P., and Nerem, R.M. Dynamic mechanical conditioning of collagen-gel blood vessel constructs induces remodeling *in vitro*. Ann Biomed Eng **28,** 351, 2000.
7. Tranquillo, R.T., Girton, T.S., Bromberek, B.A., Triebes, T.G., and Mooradian, D.L. Magnetically orientated tissue-equivalent tubes: application to a circumferentially orientated media-equivalent. Biomaterials **17,** 349, 1996.
8. L'Heureux, N., Germain, L., Labbe, R., and Auger, F.A. *In vitro* construction of a human blood vessel from cultured vascular cells: a morphologic study. J Vasc Surg **17,** 499, 1993.
9. Dahl, S.L., Koh, J., Prabhakar, V., and Niklason, L.E. Decellularized native and engineered arterial scaffolds for transplantation. Cell Transplant **12,** 659, 2003.
10. Robotin-Johnson, M.C., Swanson, P.E., Johnson, D.C., Schuessler, R.B., and Cox, J.L. An experimental model of small intestinal submucosa as a growing vascular graft. J Thorac Cardiovasc Surg **116,** 805, 1998.
11. Badylak, S.F., Record, R., Lindberg, K., Hodde, J., and Park, K. Small intestinal submucosa: a substrate for *in vitro* cell growth. J Biomater Sci Polym Ed **9,** 863, 1998.
12. Schmidt, C.E., and Baier, J.M. Acellular vascular tissues: natural biomaterials for tissue repair and tissue engineering. Biomaterials **21,** 2215, 2000.
13. Simon, P., Kasimir, M.T., Seebacher, G., Weigel, G., Ullrich, R., Salzer-Muhar, U., Rieder, E., and Wolner, E. Early failure of the tissue engineered porcine heart valve SYNERGRAFT in pediatric patients. Eur J Cardiothorac Surg **23,** 1002–1006; discussion 1006, 2003.
14. L'Heureux, N., Paquet, S., Labbe, R., Germain, L., and Auger, F. A completely biological tissue-engineered human blood vessel. FASEB J **12,** 47, 1998.
15. Michel, M., L'Heureux, N., Pouliot, R., Xu, W., Auger, F.A., and Germain, L. Characterization of a new tissue-engineered human skin equivalent with hair. In Vitro Cell Dev Biol Anim **35,** 318, 1999.
16. Germain, L., Auger, F.A., Grandbois, E., Guignard, R., Giasson, M., Boisjoly, H., and Guerin, S.L. Reconstructed human cornea produced *in vitro* by tissue engineering. Pathobiology **67,** 140, 1999.
17. Nerem, R.M. Tissue engineering a blood vessel substitute: the role of biomechanics. Yonsei Med J **41,** 735, 2000.
18. Laflamme, K., Roberge, C.J., Grenier, G., Remy-Zolghadri, M., Pouliot, S., Baker, K., Labbe, R., D'Orleans-Juste, P., Auger, F.A., and Germain, L. Adventitia contribution in vascular tone: insights from adventitia-derived cells in a tissue-engineered human blood vessel. FASEB J **20,** 1245, 2006.
19. Grenier, G., Remy-Zolghadri, M., Guignard, R., Bergeron, F., Labbe, R., Auger, F.A., and Germain, L. Isolation and culture of the three vascular cell types from a small vein biopsy sample. In Vitro Cell Dev Biol Anim **39,** 131, 2003.
20. Pricci, M., Bourget, J.M., Robitaille, H., Porro, C., Soleti, R., Mostefai, H.A., Auger, F.A., Martinez, M.C., Andriantsitohaina, R., and Germain, L. Applications of human tissue-engineered blood vessel models to study the effects of shed membrane microparticles from T-lymphocytes on vascular function. Tissue Eng Part A **15,** 137, 2009.
21. Berglund, J.D., Nerem, R.M., and Sambanis, A. Incorporation of intact elastin scaffolds in tissue-engineered collagen-based vascular grafts. Tissue Eng **10,** 1526, 2004.
22. Berglund, J.D., Nerem, R.M., and Sambanis, A. Viscoelastic testing methodologies for tissue engineered blood vessels. J Biomech Eng **127,** 1176, 2005.
23. Fung, Y.C. Biomechanics: Mechanical Properties of Living Tissues, 2nd edition. New-York: Springer-Verlag, 1993.
24. Burton, A.C. Relation of structure to function of the tissues of the wall of blood vessels. Physiol Rev **34,** 619, 1954.
25. Powell, R.J., Hydowski, J., Frank, O., Bhargava, J., and Sumpio, B.E. Endothelial cell effect on smooth muscle cell collagen synthesis. J Surg Res **69,** 113, 1997.
26. Stegemann, J.P., and Nerem, R.M. Phenotype modulation in vascular tissue engineering using biochemical and mechanical stimulation. Ann Biomed Eng **31,** 391, 2003.
27. Fu, P., Sodian, R., Luders, C., Lemke, T., Kraemer, L., Hubler, M., Weng, Y., Hoerstrup, S.P., Meyer, R., and Hetzer, R. Effects of basic fibroblast growth factor and transforming growth factor-beta on maturation of human pediatric aortic cell culture for tissue engineering of cardiovascular structures. ASAIO J **50,** 9, 2004.
28. Sales, V.L., Engelmayr, G.C., Jr., Mettler, B.A., Johnson, J.A., Jr., Sacks, M.S., and Mayer, J.E., Jr. Transforming growth factor-beta1 modulates extracellular matrix production, proliferation, and apoptosis of endothelial progenitor cells in tissue-engineering scaffolds. Circulation **114,** I193, 2006.
29. Cummings, C.L., Gawlitta, D., Nerem, R.M., and Stegemann, J.P. Properties of engineered vascular constructs made from collagen, fibrin, and collagen-fibrin mixtures. Biomaterials **25,** 3699, 2004.
30. Isenberg, B.C., and Tranquillo, R.T. Long-term cyclic distention enhances the mechanical properties of collagen-based media-equivalents. Ann Biomed Eng **31,** 937, 2003.
31. Berglund, J.D., Mohseni, M.M., Nerem, R.M., and Sambanis, A. A biological hybrid model for collagen-based tissue engineered vascular constructs. Biomaterials **24,** 1241, 2003.
32. Guillemette, M.D., Cui, B., Roy, E., Gauvin, R., Giasson, C.J., Esch, M.B., Carrier, P., Deschambeault, A., Dumoulin, M., Toner, M., Germain, L., Veres, T., and Auger, F.A. Surface topography induces 3D self-orientation of cells and

extracellular matrix resulting in improved tissue function. Integr Biol **1,** 196, 2009.
33. Nerem, R.M. Role of mechanics in vascular tissue engineering. Biorheology **40,** 281, 2003.
34. Iwasaki, K., Kojima, K., Kodama, S., Paz, A.C., Chambers, M., Umezu, M., and Vacanti, C.A. Bioengineered three-layered robust and elastic artery using hemodynamically-equivalent pulsatile bioreactor. Circulation **118,** S52, 2008.
35. Laflamme, K., Roberge, C.J., Labonte, J., Pouliot, S., D'Orleans-Juste, P., Auger, F.A., and Germain, L. Tissue-engineered human vascular media with a functional endothelin system. Circulation **111,** 459, 2005.
36. Grenier, G., Remy-Zolghadri, M., Bergeron, F., Guignard, R., Baker, K., Labbe, R., Auger, F.A., and Germain, L. Mechanical loading modulates the differentiation state of vascular smooth muscle cells. Tissue Eng **12,** 3159, 2006.
37. Konig, G., McAllister, T.N., Dusserre, N., Garrido, S.A., Iyican, C., Marini, A., Fiorillo, A., Avila, H., Wystrychowski, W., Zagalski, K., Maruszewski, M., Jones, A.L., Cierpka, L., de la Fuente, L.M., and L'Heureux, N. Mechanical properties of completely autologous human tissue engineered blood vessels compared to human saphenous vein and mammary artery. Biomaterials **30,** 1542, 2008.
38. Kanda, K., Matsuda, T., and Oka, T. Two-dimensional orientational response of smooth muscle cells to cyclic stretching. ASAIO J **38,** M382, 1992.
39. Grenier, G., Remy-Zolghadri, M., Larouche, D., Gauvin, R., Baker, K., Bergeron, F., Dupuis, D., Langelier, E., Rancourt, D., Auger, F.A., and Germain, L. Tissue reorganization in response to mechanical load increases functionality. Tissue Eng **11,** 90, 2005.
40. Syedain, Z.H., Weinberg, J.S., and Tranquillo, R.T. Cyclic distension of fibrin-based tissue constructs: evidence of adaptation during growth of engineered connective tissue. Proc Natl Acad Sci U S A **105,** 6537, 2008.
41. Xu, Z.C., Zhang, W.J., Li, H., Cui, L., Cen, L., Zhou, G.D., Liu, W., and Cao, Y. Engineering of an elastic large muscular vessel wall with pulsatile stimulation in bioreactor. Biomaterials **29,** 1464, 2008.

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