REVIEW ARTICLE

Small Diameter Xenogeneic Extracellular Matrix Scaffolds for Vascular Applications

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Currently, despite the success of percutaneous coronary intervention (PCI), coronary artery bypass graft (CABG) remains among the most commonly performed cardiac surgical procedures in the United States. Unfortunately, the use of autologous grafts in CABG presents a major clinical challenge as complications due to autologous vessel harvest and limited vessel availability pose a significant setback in the success rate of CABG surgeries. Acellular extracellular matrix (ECM) scaffolds derived from xenogeneic vascular tissues have the potential to overcome these challenges, as they offer unlimited availability and sufficient length to serve as “off-the-shelf” CABGs. Unfortunately, regardless of numerous efforts to produce a fully functional small diameter xenogeneic ECM scaffold, the combination of factors required to overcome all failure mechanisms in a single graft remains elusive. This article covers the major failure mechanisms of current xenogeneic small diameter vessel ECM scaffolds, and reviews the recent advances in the field to overcome these failure mechanisms and ultimately develop a small diameter ECM xenogeneic scaffold for CABG.

Keywords: extracellular matrix, xenogeneic scaffolds, vessel scaffold, small diameter vessel, xenogeneic small diameter vessel

Impact Statement

Currently, the use of autologous vessel in coronary artery bypass graft (CABG) is common practice. However, the use of autologous tissue poses significant complications due to tissue harvest and limited availability. Developing an alternative vessel for use in CABG can potentially increase the success rate of CABG surgery by eliminating complications related to the use of autologous vessel. However, this development has been hindered by an array of failure mechanisms that currently have not been overcome. This article describes the currently identified failure mechanisms of small diameter vascular xenogeneic extracellular matrix scaffolds and reviews current research targeted to overcoming these failure mechanisms toward ensuring long-term graft patency.

Introduction

CARDIOVASCULAR DISEASE (CVD) is the leading cause of death worldwide, accounting for 17 million deaths annually, with >600,000 deaths in the United States per year.^{1–4} Over 50% of these deaths are caused by atherosclerotic vascular disease (AVD) particularly in wealthy, developed countries where dietary factors promote disease development.^{4–7} AVD is characterized by the chronic deposition of subendothelial atheromatous plaque, affecting the coronary, cerebral, and/or peripheral arteries.⁷ Coronary AVD causes >7.2 million deaths worldwide every year due to acute coronary syndrome (ACS).⁸ ACS occurs when an AVD lesion results in critical vessel stenosis and/or rupture of a vulnerable plaque, leading to compromised blood flow and resultant is-

chemia of the myocardial territory supplied by the culprit vessel.⁹ Rapid revascularization is critical to minimize irreversible myocardial damage in ACS patients.¹⁰

Despite the success of percutaneous coronary intervention (PCI), CABG remains among the most commonly performed cardiac surgical procedures in the United States, with >400,000 procedures performed every year.^{11,12} American College of Cardiology guidelines provide a class I indication for emergency coronary artery bypass graft (CABG) in patients with left main and/or 3-vessel disease, failed PCI, coronary anatomy not amenable to PCI, mechanical complication of ST-elevation myocardial infarction, or cardiogenic shock.¹³ In addition, the success of PCI in uncomplicated cases has increased CABG case complexity and number of grafts employed per patient, with current AHA statistics indicating

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an average of 2.6 grafts utilized per patient necessitating harvest from more than one vessel in the majority of CABG patients.^{13–18}

Currently, autologous vessels serve as the main source of grafts for CABG surgery. Autologous venous and arterial grafts have been utilized from a variety of locations, including the left and right internal mammary artery (LIMA and RIMA, respectively), radial artery (RA), gastroepiploic artery (GEA), and saphenous vein (SV).¹⁹ Differences in harvesting techniques, anatomy, and physiology have all been implicated as factors, which influence postimplantation graft patency rates (Table 1).^{14,20–23} The highest 10-year patency rates are reported for LIMA grafts (90–95%), followed by RA (80%), RIMA (72%), GEA (62.5%), and finally the SV (50%).^{14,15,24,25}

Arterial conduit harvest has been associated with donor site morbidity and long-term complications, such as upper limb or breast ischemia, impaired respiratory function, and increased risk of sternal complications. Consequently SV grafts are still frequently utilized, despite inferior patency rates.^{21,26–29} In addition, the length and availability of SV make it an attractive conduit for CABG surgery.²³ CABG surgery reliance on SV grafts has encouraged researchers to propose alternatives that promote SV graft patency. Damage to the endothelium during vessel harvest has been shown to affect short- and long-term SV graft patency, prompting development of the “no-touch technique” for SV harvest.²³ This technique is routinely used for arterial conduit harvest, but inexplicably, only recently used for venous conduits.^{16,20,21}

The no-touch technique aims to harvest the SV in an atraumatic manner by including a pedicle of tissue surrounding the vessel during harvest and avoiding vessel distention during flushing. The no-touch technique increased patency of SV grafts to 90% at 8.5 years, comparable with that of the internal mammary arteries.²³ Despite such advances in surgical technique, use of autologous SV still has the potential to result in donor site complications such as limb ischemia, cellulitis, neuropathy, wound infections, and nonhealing wounds.^{30–32} In addition, regardless of harvest site, peripheral vascular disease, amputation, previous vessel harvest, or limited vessel length continue to pose challenges to the use of autologous vessels for CABG.³³

Acellular extracellular matrix (ECM) scaffolds derived from xenogeneic vascular tissues have the potential to overcome the challenges associated with autologous vessel harvest in CABG. Vascular ECM scaffolds offer sufficient length and unlimited availability, eliminating the need for donor vessel harvest and its associated complications. Fur-

thermore, vascular ECM scaffolds afford several potential advantages over synthetic alternatives. For instance, the use of animal tissue for the production of ECM provides researchers with an immense array of options to successfully match size, length, and compliance between donor vessel and recipient vessel; possibly eliminating one of the main failure mechanisms for synthetic small diameter grafts (i.e., <5 mm).^{34–39}

The complex multiscalar structure, composition, architecture, and resultant function of native ECM are challenging to fully recapitulate with synthetic approaches. Indeed, the native ECM environment of xenogeneic scaffolds has been shown to modulate cell adhesion, migration, and proliferation, driving proregenerative recipient cellular repopulation and tissue-specific cellular differentiation.^{34–39} Consequently, vascular ECM scaffolds have potential to revolutionize CABG as they provide the field with an alternative graft source, which recapitulates the properties of native vascular ECM, modulates repopulating cell behavior, and thereby has potential to overcome the challenges associated with current autologous vessel use.

Glutaraldehyde (GTA)-fixed xenogeneic tissues have been widely used in clinical practice since the 1960s, with the most common application being heart valves.^{39,40–43} Although GTA-fixed xenogeneic vascular grafts have been utilized successfully for large diameter vessel applications such as arteriovenous fistulas and lower extremity bypasses, they have failed to translate to small diameter vascular applications since the primary failure modes of GTA-fixed tissues are poorly tolerated in small diameter vessels (see Failure Mechanisms section).^{44–48}

GTA is a nonreversible crosslinking agent used to limit immune-mediated surveillance of xenogeneic tissues. Cross-linking achieved with GTA fixation also improves tissue durability, slowing xenogeneic tissue degradation rate.⁴⁹ While broadly used, GTA fixation is also associated with alterations to mechanical properties, toxicity, and tissue calcification, reducing the long-term functionality of such biomaterials and vascular graft patency rates in particular.^{50–52} Furthermore, the nonreversible nature of GTA fixation prevents recipient cellular repopulation, proregenerative ECM signaling, and associated remodeling. Consequently, although use of GTA fixation mitigates acute graft-specific adaptive immune-mediated destruction of xenogeneic tissues, negative consequences of GTA use limit the regenerative potential of such materials.

The field of xenogenic vascular grafts has moved toward alternative tissue processing techniques (i.e., decellularization,

TABLE 1. ANATOMICAL AND PHYSIOLOGICAL COMPARISON OF AUTOLOGOUS VESSEL TISSUE USED IN CORONARY ARTERY BYPASS

Vessel	Physiology					
	Inner diameter (mm)	Wall thickness (μm)	Length (cm) ⁴⁰	Muscular or elastic	Tendency to spasm	Nitric oxide production
Coronary artery	2 ± 0.45 ⁴⁰	258 ± 41 ⁴⁰	7.54 ± 1.84	Muscular ⁴⁰	—	—
Internal mammary arteries	1.6 ± 0.3 ⁴¹	200 ± 67 ⁴²	19.48 ± 3.57	Elastic ⁴³	Low ¹⁴	High ¹⁴
Radial artery	2.4 ± 0.3 ⁴⁴	290 ± 63 ⁴⁴	22.28 ± 3.31	Muscular ⁴³	Highest ⁴⁵	
Gastroepiploic artery	2.7 ± 0.3 ⁴⁶	197 ± 77 ⁴⁰	19.32 ± 4.18	Muscular ⁴³	High ⁴⁷	
Saphenous vein	3.9 ± 0.45 ⁴⁸	200 ± 3 ⁴⁰	72.42 ± 6.6	—	—	Low

antigen removal [AR]) designed to eliminate antigenic components of vascular tissues, while maintaining the positive proregenerative properties of native ECM. Unfixed xenogeneic ECM scaffolds have been used for several applications with great success (e.g., pericardial patches, hernia repair, large diameter vascular patches), but have never been successfully used as small diameter vessel grafts in humans.^{40–43,53–55} Failure to translate successes of unfixed xenogeneic ECM scaffolds in noncritical sites to vascular application results from the rigorous structural, compositional, and functional requirements of small diameter vessels.

This systematic review focuses on the current state of the art for small diameter vascular ECM scaffolds for CABG applications, highlighting previously reported failure mechanisms of such biomaterials and novel research that has potential to resolve these issues. Even though this review is focused on CABG only, such grafts may have other widespread surgical applications.

Failure Mechanisms

As interest in production of unfixed xenogeneic vascular grafts increases, the field has identified an array of failure mechanisms that need to be resolved before the promise of small diameter xenogeneic ECM scaffolds is achieved. These failure mechanisms include thrombosis, immune-mediated rejection, aneurysm, intimal hyperplasia, and scaffold calcification (Fig. 1).^{56–60} Each of these failure mechanisms is capable of leading to catastrophic conduit failure. Consequently, overcoming all of these failure mechanisms is critical to achieving long-term *in vivo* functionality. Understanding the failure mechanisms responsible for poor outcome in previously reported xenogeneic graft studies has potential to guide ongoing research efforts to overcome these challenges in small diameter vascular ECM scaffolds.

This review examines the previously reported failure mechanisms for xenogeneic vascular ECM grafts, and highlights recent research that strives to overcome these

FIG. 1. Timeline of failure mechanisms reported for xenogeneic ECM scaffolds. Factors (*red text*) previously described to initiate individual failure mechanisms, and proposed *in vitro* mechanisms (*blue text*), which must be considered during scaffold generation to overcome *in vivo* failure. ECM, extracellular matrix.

challenges. Where available, discussion will be limited to vascular graft applications, however, in instances where primary peer-reviewed manuscripts focusing on vascular grafts are unavailable, research from other tissues and implantation sites will be considered.

Vessel Thrombosis

Thrombosis is an extremely commonly reported failure mechanism of vascular ECM scaffolds (Fig. 2). Some degree of thrombosis was evident in all manuscripts that specifically examined vascular ECM scaffold thrombogenicity (18/18), although the extent of thrombus formation varied between reports.^{61–78} Thrombosis results in reduced blood flow due to obstruction of the graft lumen, and in extreme cases can lead to complete graft occlusion. Furthermore, once formed thrombi are at risk of fragmentation leading to partial or total occlusion of target organ vessels.

The role of a healthy quiescent endothelium in thrombosis and hemostasis has been reviewed elsewhere.⁷⁹ In brief, endothelial cells (ECs) in native vasculature play an important role in vessel homeostasis by maintaining a dynamic equilibrium between antithrombotic and prothrombotic surfaces depending on the current physiological need of the vessel.⁸⁰ This equilibrium is maintained by endothelial secretion of a wide array of proteins with either antithrombogenic (e.g., thrombomodulin, protein S, endothelial surface heparin sulfate, tissue factor pathway inhibitor) or prothrombotic (e.g., Von Willebrand factor [vWF]) effects.^{79,81,82} Moreover, ECs inhibit activation of both the primary (i.e., platelet adhesion and activation) and secondary (i.e., coagulation cascade) hemostatic pathways by acting as a physical barrier separating platelets and coagulation factors from subendothelial proteins (e.g., collagen, elastin, tissue factor).⁸⁰

In xenogeneic ECM scaffolds, the risk of thrombosis is exacerbated by the lack of ECs coverage regardless of scaffold processing method. Absence of ECs in xenogeneic scaffolds results in lack of production of antithrombotic proteins, and eliminates the physical barrier that separates ECM proteins from platelets and coagulation factors.⁶⁷ For example, lack of ECs exposes subendothelial vWF, which facilitates platelet adhesion through the GPIIb/IIIa and

GP1b receptors. Unfortunately, even if all subendothelial vWF is removed by the tissue processing method, plasma vWF can still bind to exposed collagen in denuded tissue and initiate primary thrombus formation.⁸¹ Similarly, the lack of ECs barrier also exposes structural ECM proteins, which serve as binding sites for platelet glycoprotein VI (GPVI).

GPVI is a platelet membrane protein known to be a receptor for collagen and laminin.^{83,84} Collagen-bound GPVI initiates a signaling cascade that results in platelet activation and ultimately activation of the coagulation cascade.⁸¹ Laminin-bound GPVI also activates platelets, although to a lesser extent, and requires integrin-mediated laminin–platelet binding.⁸⁴ The importance of providing an antithrombotic barrier between the blood stream and exposed ECM proteins has driven a number of approaches to reduce the thrombogenic potential of vascular ECM scaffolds. *In vitro* ECs seeding, coating of luminal surface with anticoagulant compounds (e.g., heparin), and fostering rapid *in vivo* re-endothelialization have all been examined as methods to reduce the risk of vascular ECM scaffold thrombosis (see overcoming failure mechanisms section).^{71,76,85,86}

ECM Scaffold Rejection

ECM scaffold rejection is possibly the most challenging failure mechanism for the field to solve. The mechanisms by which the immune system reacts to ECM materials are reviewed in detail elsewhere.⁸⁷ In brief, an ideal tissue processing method would eliminate all tissue antigens or at least reduce their content below the threshold required for adaptive immune activation, while maintaining a resultant vascular ECM scaffold structure, composition, and function.⁸⁸ Most current tissue processing methods are capable of eliminating enough antigens to reduce the host immune response toward the ECM scaffold to some degree, but are rarely capable of eliminating it completely.^{60,89–91} ECM scaffold rejection is the process in which a transplant tissue is attacked by the immune response of the recipient (Fig. 3). This response can be provoked by different immune response pathways, which are largely orchestrated by two types of antibodies: (i) natural antibodies and (ii) acquired antibodies.⁹²

FIG. 2. Factors capable of inducing xenogeneic ECM vascular scaffold thrombosis. Achieving proper endothelialization has the potential to overcome the two known thrombosis inducing factors.

FIG. 3. Factors capable of inducing xenogeneic ECM vascular scaffold rejection. Reducing damage to the ECM matrix during *in vitro* scaffold generation has the potential to reduce destructive proinflammatory immune responses while fostering proregenerative innate immune responses. Reducing the antigenic content of ECM scaffolds avoids stimulation of destructive graft-specific adaptive immune responses.

- (i) Natural antibodies—Natural or spontaneous antibodies are present in the body even in the absence of any prior direct exposure to their antigens.⁹³ Natural antibodies are acquired by indirect exposure to their antigens through the environment.⁹⁴ The most relevant case for xenotransplantation is the generation of antibodies against the oligosaccharide galactose- α -1,3-galactose (α -Gal) in humans due to continuous exposure to the α -gal moiety on mucosal surfaces of the digestive system.⁸⁸ The topic of natural antibodies in xenotransplantation is reviewed in detail elsewhere.⁹⁵
- (ii) Acquired antibodies—Acquired antibodies are produced by antigen-specific B-cells after exposure to their antigens. However, isotype switching and maturation of B-cell antibody production require T-helper cell help. Consequently, production of epitope-specific IgG toward highly immunogenic antigens requires a cascade of events involving interaction between the cell-mediated and humoral arms of the adaptive immune system.^{92,96,97} This topic is reviewed in detail elsewhere.⁹⁸

Regardless of the type of antibody response, failure to eliminate antigens results in increased risk of scaffold immune destruction. Antibodies bind to remnant antigens in xenogeneic scaffolds, initiating recruitment of both adaptive and innate immune cells.⁸⁷ Immune cell recruitment is further exacerbated by the presence of graft chemoattractants, such as collagen fragments.^{57,99} Antigen recognition and costimulation between cells result in increased secretion of cytokines and matrix metalloproteinase (MMP).^{100,101} Cytokines further stimulate a proinflammatory response, cell recruitment, and MMP secretion.¹⁰² Increased secretion of MMP, as mentioned previously, degrades structural proteins of the ECM scaffold leading to graft damage. The combined effect of immune activation results in the degradation of ECM proteins and scaffold failure by any or all of the aforementioned mechanisms.

Aneurysm

Aneurysmal dilation has commonly been reported as a failure mechanism for vascular ECM scaffolds. Aneurysm

is likely caused by differences in compliance between the native vessel and the ECM scaffold, and/or a proinflammatory immune response.¹⁰³ Aneurysm formation results in a number of potential complications, including increased thrombosis risk due to turbulent blood flow and potential for vessel rupture due to increased wall stress at the aneurysmal site (Fig. 4).

- (i) Compliance mismatch—Differences in mechanical properties between the CABG graft and target vessel can lead to aneurysm formation in either the native vessel or xenogeneic ECM scaffolds.¹⁰³ Increase in graft or native vessel diameter inherently increases wall tension, leading to potential for progressive aneurysmal dilation and increased risk of vessel rupture.¹⁰⁴ ECM scaffold with lower compliance than native vasculature may cause a preferential dilation of the native vessel to occur.^{104,105} Conversely, ECM scaffolds with higher compliance than the target vessel dilate under physiologic pressure, leading to potential for turbulent flow and ultimately increasing risk of target vessel dilation near the anastomosis site.¹⁰⁴

ECM scaffolds generation methods seek to avoid disruption of the native vessel mechanical properties, and thereby prevent compliance mismatch between the graft and native recipient vessel. Consequently, avoiding disruption of native ECM mechanical properties and matching those to the recipient vessel mechanical properties are essential to avoid aneurysmal failure. In instances where minor mismatches occur, cell repopulation and ECM remodeling may be capable of achieving matching of mechanical properties between the recipient vessel and the graft in the intermediate to long term. Furthermore, matrix turn over by repopulation cells has potential for stress adaptation ultimately matching graft and target vessel compliance.

Fostering rapid *in vivo* cellular repopulation for such acellular biomaterials is critical to reduce risk of early aneurysm formation. Alternatively, *in vitro* cellular repopulation may be required to normalize resultant composite biomaterial compliance before *in vivo* implantation. In the

FIG. 4. Factors capable of inducing aneurysm in xenogeneic ECM vascular scaffold. Avoiding damage to the ECM during *in vitro* scaffold generation has potential to reduce aneurysm formation due to mechanical mismatch and avoids activation of proinflammatory innate immune responses. Reducing scaffold antigenic content has potential to reduce aneurysm formation by avoiding stimulation of destructive graft-specific adaptive immune responses and associated recruitment of innate immune cells (e.g., macrophages).

case of acellular xenogeneic vascular ECM scaffolds, aneurysms may occur due to inability of the graft to resist arterial pulsatile pressures resulting in excessive graft dilation, increased wall stress, and ultimately rupture.¹⁰²

- (ii) Proinflammatory immune response—aneurysms are extremely common even in ECM scaffolds with similar burst strengths as their native target vessel.⁵⁹ In this case, progressive aneurysm formation is thought to occur due to immune-mediated degradation of ECM scaffold structural proteins. Similar to calcification, macrophages activation by the adaptive and/or the innate immune response secrete MMPs that degrade ECM scaffold's collagen and elastin, weakening the scaffold significantly, thereby increasing graft compliance.¹⁰⁶

Intimal Hyperplasia

Intimal hyperplasia is a common failure mechanism for both allograft CABG grafts near the anastomosis site and for vascular xenogeneic ECM scaffolds that avoid the previously discussed acute failure mechanisms.^{58,64,77,85,105,107–109} Intimal hyperplasia occurs due to luminal smooth muscle

cell proliferation and ECM protein deposition, resulting in thickening of the neovascular neointima (Fig. 5).¹¹⁰ In the case of allograft vessels, damage to the endothelium by surgical procedure, turbulent flow, and/or cyclical strain at the anastomosis sites all modulate alterations in the balance between ECs anti- versus proproliferative signaling, ultimately stimulating vascular smooth muscle cell (VSMC) proliferation.^{66,105,111} Decreased ECs expression of growth-inhibitory factors (e.g., nitric oxide and natriuretic peptides) and increased expression of growth-stimulating factors (e.g., fibroblast growth factor, angiotensinogen, angiotensin, platelet-derived growth factor [PDGF]) result in VSMC proliferation.⁵⁶ In addition, excessive cyclical strain due to compliance mismatch has been demonstrated to directly stimulate VSMC activation and proliferation.^{56,105}

Cell proliferation is initiated in the media, where newly divided VSMCs increase production of ECM proteins by four- to fivefold.⁵⁶ Disruption of the native ECM, along with increased expression of growth-stimulating factors, induces synthesis of plasminogen activators, which in turn degrade the ECM and activate MMPs.⁵⁶ Medial VSMCs, promoted by PDGF, migrate into the intima through the degraded ECM that no longer acts as a barrier. After intimal translocation, VSMC expansion in the intima occurs due to a

FIG. 5. Factors capable of inducing intimal hyperplasia in xenogeneic ECM vascular scaffold. Avoiding damage to the ECM matrix during *in vitro* scaffold generation has the potential to reduce hyperplasia formation, as it reduces ECM matrix degradation by MMPs. Eliciting proper endothelialization balances proliferative signals in the ECM and avoids cell overgrowth. MMP, matrix metalloproteinase.

combination of VSMC proliferation and migration, accompanied by excessive ECM synthesis.⁵⁶

Intimal hyperplasia has frequently been reported as a failure mechanism for ECM scaffolds, although the mechanism responsible for its development in the absence of medial VSMCs and its dependency on scaffold generation method remains unclear.^{58,66,109} Transformation of repopulating fibroblasts to smooth muscle phenotype has been proposed as one possible mechanism for development of intimal hyperplasia in acellular ECM scaffolds. However, the extent to which repopulating ECs mediate such transformations remains to be determined. Therefore, it would be beneficial for the field to identify common mechanisms responsible for stimulating intimal hyperplasia development between allografts and ECM scaffolds. Targeting such mechanisms may have potential to reduce risk of intimal hyperplasia in xenogeneic grafts.

In ECM scaffolds, compliance mismatch may be an important cause for intimal hyperplasia that could be mitigated by carefully selecting donor species, donor site, and tissue processing method.¹¹² Similarly, the extent to which differing ECM scaffold generation methods result in scaffolds, which are capable of fostering rapid, quiescent, endothelial monolayer formation, remains unknown and may be critical in avoiding aberrant ECs proliferative signaling and resultant smooth muscle proliferation.¹⁰⁹

Vessel Calcification

Calcification plays an important role in failure of current clinically utilized GTA-fixed xenogeneic tissues (e.g., heart valves or vascular patches). In the case of heart valves for instance, calcification is present in up to 60% of patients by 10 years, representing an important cause for reoperation.¹¹³ In the field of unfixed xenogeneic vascular ECM scaffolds the risk of calcification, leading to reduced graft patency, is well documented.^{60,91,114–116} Unfortunately, the majority of current research articles fail to test and report *in vivo* scaffold calcification potential. This lack of information leaves a gap in knowledge regarding the impact of calcification on small diameter vascular ECM scaffolds. However, insight can be achieved by interpolating calcification data for xe-

nogeneic ECM scaffolds implanted in other sites, and the results do not look promising. Regardless of tissue processing method, calcification of xenogeneic scaffolds seems to be comparable with that of GTA-fixed tissues.^{60,91,114–116} These results suggest that calcification is likely to represent an important long-term failure mechanism for vascular ECM scaffold grafts, which may be independent of the method used to produce the scaffold. It is, therefore, valuable to understand the mechanisms responsible for mediating ECM scaffold calcification, to inform development of processing methods, which may be capable of avoiding vascular ECM scaffold calcification.

Calcification is the pathologic deposition of large insoluble calcium salts in soft tissue. Calcification results in alterations in tissue mechanical properties, including increase in stiffness and concomitant reduction in vascular compliance.^{117–119} The exact mechanisms of xenogeneic tissues calcification are not completely understood; however, several theories have been proposed to explain ECM scaffold calcification: (i) organic matrix composition, (ii) graft-specific immune response, (iii) presence of lipids, and (iv) mechanical stress (Fig. 6).^{120,121}

- (i) Organic matrix composition—ECM is inherently susceptible to development of calcification due to the high affinity of collagen and particularly elastin to calcium ions.¹¹³ According to the charge neutralization theory, calcium ions bind to neutrally charged binding sites in the peptide chain backbone of elastin molecules.¹²² Once the elastin loci are positively charged, they attract charge-neutralizing ions such as phosphate and carbonate. Upon site neutralization, adjacent loci in the peptide backbone are available for calcium binding and subsequent neutralization, until the crystallization process is initiated. Calcium binding to the backbone of a peptide chain, especially if shared by two chains, results in stiffening of the protein structure and reduced elasticity.¹²²

In healthy vessels, an array of mineralization-regulating proteins (e.g., osteopontin, osteoprotegerin, matrix Gla protein, and osteocalcin) are responsible for inhibiting elastin

FIG. 6. Factors capable of inducing calcification in xenogeneic ECM vascular scaffold. Research in small diameter vessel xenogeneic ECM is currently focused on three main areas that have the potential of overcoming identifiable failure mechanisms: (i) reducing graft antigenic content, (ii) avoiding any damage to the ECM scaffold, and (iii) eliciting proper endothelialization. Optimization of each of these areas has the potential to avoid calcification by targeting different calcification inducing factors.

calcification.^{123–133} Due to their high calcium affinity, such mineralization-regulating proteins effectively quench free calcium ions preventing its deposition on elastin.¹³⁴ The extent to which current tissue processing methods (e.g., decellularization or AR) retain or remove the mineralization-regulating proteins remains largely uninvestigated. Consequently, the extent to which retention of mineralization-regulation proteins in vascular ECM scaffolds may reduce or eliminate *in vivo* calcification is unknown.

In native tissue, smooth muscle and ECs are the primary cell types responsible for secretion of mineralization-regulating proteins. Therefore, recellularization of xenogeneic ECM vascular scaffolds, either *in vitro* or *in vivo*, has potential to serve as a route to replace mineralization-regulating proteins lost during tissue processing.^{124,135} Retention or reconstitution of mineralization-regulating proteins in vascular ECM scaffolds is therefore likely to be critical in mitigating elastin-mediated calcification.

- (ii) Graft-specific immune response—Xenogeneic ECM scaffolds are subject to both adaptive and innate immune surveillance.^{88,116} Antigens left in the tissue by ineffective tissue processing methods stimulating adaptive responses, while disruption of native ECM protein macromolecular structure promotes detrimental innate immune responses.^{88,89,91,116,136–138} Residual antigenic epitopes in ECM scaffolds are subject to recipient antigen-specific T and/or B cell recognition. Once activated, cells of the adaptive immune system produce cytokines, chemokines, and antibodies, promoting recruitment and activation of both additional adaptive and innate (e.g., macrophages) immune cells.^{139,140}

The innate immune system has potential to recognize patterns of altered ECM protein macromolecular structure and initiate an immune response that also ultimately results in macrophage activation.^{141–143} Activated macrophages secrete MMPs, a group of enzymes responsible for ECM remodel by protein degradation.^{144,145} MMP-degraded ECM elastin exhibits a significant increase in their calcium ion binding affinity, resulting in an increase in the number of nucleation sites available for calcification.^{146–148} Consequently, regardless of the inciting cause for immune recognition (i.e., adaptive or innate recognition) convergence and recruitment of both arms of the immune system results in macrophage activation, matrix degradation, nucleation site exposure, and increased risk of calcification.

- (iii) Presence of Lipids—Calcification due to lipid content in ECM scaffolds has been shown to occur in both GTA-fixed and unfixed xenogeneic tissues.^{142,149,150} Lipid-mediated calcification is thought to occur due to interaction between high-density negatively charged phospholipids and calcium ions within the plasma.^{147,151} During dystrophic calcification, lipids act as mineralization nucleators, causing precipitation of saturated calcium phosphate leading to tissue calcification.^{152,153} While several research articles have demonstrated a high correlation between lipid content and calcification in GTA-fixed tissue, this correlation has not been as clearly demonstrated for unfixed ECM scaffolds.^{152,154}

Some evidence suggests that decellularization methods using cell lysis, enzymatic digestion, and detergent treatment are inefficient at lipid removal, and residual lipid content correlates with *in vivo* scaffold calcification.^{142,149} However, other articles have reported a complete absence of calcification of decellularized tissues in *in vivo* animal models.¹⁴² AR methods incorporating specific lipophile extraction steps have been reported to significantly reduce resultant scaffold lipid content and ameliorate *in vivo* calcification.⁹¹ Consequently, the extent to which residual lipids contribute to nonfixed ECM scaffolds calcification remains unclear and requires further investigation. Greater understanding of the role of residual lipids in ECM scaffold calcification has potential to predict *in vivo* calcification response and thereby guide ECM scaffold production endeavors.

Mechanical Stress—Calcification at leaflet attachment points is a common failure mode for GTA-fixed heart valve prosthesis.^{155,156} This calcification is frequently seen along the zones of high compressive and tensile stresses, leading to the hypothesis that mechanical stress serves as a direct cause of calcification in GTA-fixed valves.¹⁵⁷ These mechanical stresses result from differences in material properties between host tissue and the implanted ECM scaffold (compliance mismatch), which lead to stress concentration at sites of cyclic loading of the ECM scaffold.^{156,158–160} This interfacial stress concentration and cyclic loading cause degradation of ECM structural components, exposure of nucleation sites, and subsequent calcification.^{132,161,162}

Compliance mismatch is highly plausible between non-fixed vascular ECM scaffolds and host tissue. Xenogeneic vascular tissues are harvested from a range of donor species, sites of origin, and can be processed with a wide array of methods, all of which have potential to affect mechanical properties of the resultant ECM scaffold.^{163,164} Consequently, the potential for compliance mismatch at the interface with the recipients vessel exists. Conversely, calcification due to mechanical stresses is not known to happen in allografts or autografts, despite differences in compliance between the harvested graft and target vessel. Lack of calcification in autologous vessels may indicate the need for a threshold of compliance mismatch and associated mechanical stress that is exceeded in GTA-fixed tissues, but not reached in auto- or allografts. Therefore, it would be beneficial for the field to determine the compliance mismatch threshold that needs to be surpassed for mechanical stresses to be a factor in the calcification of nonfixed small diameter vessel ECM scaffolds.

Overcoming Failure Mechanisms in Vascular ECM Scaffolds

It is very attractive to use xenogeneic tissue in the field of tissue engineering due to its unlimited availability, similarities in size, composition, and function to native vessels. Therefore, efforts dedicated to solve the limitations of small diameter vascular ECM scaffolds have led to a vast array of modifications, conditionings, and chemical changes to improve the *in vivo* outcome of such biomaterials. Different techniques have resulted in varying degrees of success in mitigating one or more of the previously discussed failure modes, contributing valuable information to the field.

Researchers have focused their efforts into overcoming three main underlying causes for ECM scaffold failure: (i) graft-specific immune response, (ii) altered mechanical properties, and (iii) improper recellularization.

(i) Graft-specific immune response: Overcoming graft-specific immune response is crucial for the success of small vessel xenogeneic scaffolds, since either the adaptive or the innate immune response can elicit an array of failure mechanisms, including calcification, aneurysm, and graft rejection. A variety of approaches have been taken with the goal of overcoming recipient adaptive and innate immune response toward xenogeneic vascular grafts, and ultimately reducing any response. These approaches fall broadly into three categories: (1) antigen masking through crosslinking procedures, (2) minimally immunogenic tissue sources, and (3) improvement of tissue processing methods for antigen elimination.

Theoretically, crosslinking has the potential to ameliorate both the adaptive and innate immune responses by masking antigenic components and ECM components, which may have been disrupted during the scaffold generation process, respectively. However, traditional crosslinking methods are mainly used in the field to mask antigenic components of tissue either by direct modification of antigenic epitopes or by reducing antigen availability for adaptive immune surveillance. GTA fixation has been extensively utilized as a method to reduce recipient adaptive immune response toward xenogeneic tissues and ECM scaffolds. Although GTA fixation dramatically reduces acute adaptive immune response toward xenogeneic tissues, chronic responses persist.^{165,166} Indeed for example, in human patients receiving GTA-fixed heart valves, chronic immune responses toward 19 individual non-HLA antigens were recently reported.¹⁶⁷ Similarly, GTA-fixed heart valves explanted due to development of structural valve deterioration have been shown to be universally positive for non- α -Gal IgG presence.^{89,168}

GTA fixation is also associated with alterations in ECM scaffold mechanical properties and renders the resultant biomaterial prone to calcification due to free aldehyde residues in the resultant tissue.¹⁶⁹ As previously discussed, the specific limitations of GTA fixation are likely to be particularly detrimental in vascular tissues since they coincide with known failure mechanisms of small diameter vascular grafts. The limitations of GTA fixation have led investigators to examine other potential crosslinking agents.

Procyanidins (PCs) are a type of crosslinking agents that have been investigated by several groups in the field as a replacement of GTA fixation.^{169,170} Crosslinking with PC generates small diameter vascular ECM scaffolds capable of supporting cell growth, while being proteolysis and calcification resistant.^{169,170} However, results regarding immunogenicity of PC crosslinked ECM scaffolds are somewhat contradictory, since PC-crosslinked ECM scaffolds seem to elicit lower platelet adhesion than GTA-fixed or native tissue, but attracted the same number of immune cells (primarily phagocytes).¹⁷⁰ Furthermore, PC crosslinking caused significant changes to vascular graft mechanical properties, which could potentially enhance the risk of nonimmunogenic failure mechanisms.¹⁷⁰

Ultimately, all permanent crosslinking approaches share one potentially critical limitation, which is that they prevent incorporation and turnover of the resultant biomaterial into recipient tissues. Therefore, if crosslinking is the route of

choice to attempt to reduce ECM scaffold immunogenicity, research efforts should focus on improving upon crosslinking agents compatible with *in vivo* crosslink breakdown to allow for scaffold turnover.¹⁷¹

With the goal of identifying the ideal tissue source for minimally immunogenic response, researchers have looked into different approaches to resolve the antigenic content in animal tissue. The advancement of gene editing techniques has allowed researchers to breed α -gal knockout animals (primarily pigs), eliminating the first immunogenic barrier for xenogeneic transplantation.¹⁷² Different tissues and organs from α -gal knockout pigs have been tested *in vitro* and *in vivo*, resulting in overall lower immunogenic response than wild-type tissues and organs.^{173,174} However, although hyperacute rejection is alleviated by such grafts, due to the vast quantity of other antigenic proteins in animal tissue graft-specific acute and chronic immune responses persist, resulting in graft deterioration.^{175,176}

The maturity of donor animals has been examined in an attempt to reduce the adaptive immune response toward the xenografts. Extensive research in the use of fetal tissue for wound healing has demonstrated the superiority of fetal tissue over adult tissue to elicit regeneration, making it an attractive alternative tissue source.¹⁷⁷ In addition, it is demonstrated that the production of important antigenic proteins, such as major histocompatibility complex (MHC), is age dependent making fetal tissues an attractive tissue source for ECM scaffold generation.^{178,179}

Biocompatibility tests on fetal vessels demonstrated to be less immunogenic than the same vessel obtained from adult tissue.¹⁸⁰ Subdermal implantation of fetal xenogeneic vascular ECM scaffolds demonstrated a lower magnitude of host immune response compared with adult tissue.¹⁸⁰ Fetal ECM scaffold implants underwent less calcification, lower macrophage infiltration, and less matrix damage than their adult counterparts.¹⁸⁰ Further testing of fetal scaffolds as small diameter vessel implants also provided an array of positive results.¹⁸¹ Fetal small diameter vessel scaffolds implanted as carotid artery interposition resulted in high degree of graft patency, function, and ECM scaffold remodeling 6 months after implantation.¹⁸¹ Patency levels and function were determined by the absence of thrombi by Duplex ultrasound. Evidence of scaffold remodeling was reported based on the presence of evenly infiltrated fibroblasts within the tunica media and ECs in a continuous inner layer. In addition, rhythmic vasodilation and contraction of the neovessels were observed.¹⁸¹ However as these ECM scaffolds were seeded with autologous ECs before implantation it is difficult to determine which factor was responsible for these positive results.^{180,181}

As mentioned previously, an ECM scaffold with an EC layer has the potential to overcome several failure mechanisms, including calcification, thrombosis, and potentially macrophage infiltration. Hence, the reported results could potentially be attributed to the ECs layer seeded on the scaffolds before implantation instead of the lower antigenicity of fetal vascular tissues. Therefore, further testing is necessary to define the independent advantages of the low antigenicity of fetal tissue and to determine whether the benefits of low immunogenicity outweigh the disadvantages of an immature ECM with biomechanical and biochemical properties, which are not equivalent to those of adult tissue.^{182,183}

A different approach to overcome recipient graft-specific adaptive and immune response toward xenogeneic vascular ECM scaffolds has been to modify tissue processing methods, with the goal of reducing or eliminating resultant ECM scaffold xenoantigen content while maintaining the macromolecular structure of the remaining ECM components. Currently, decellularization represents the most commonly utilized tissue processing method for vascular ECM scaffold production. As the entirety of articles relevant for this review and the vast majority of current research in tissue engineering aim to produce scaffolds through decellularization process.

Decellularization, theoretically consists in eliminating the host immune response toward ECM scaffolds by removing exclusively cellular components, while maintaining the structural integrity and function of ECM proteins (i.e., collagen, elastin, laminin).¹⁸⁴ However, an important distinction has been highlighted between absence of cellular elements and elimination of immunogenic antigens. Existence of noncellular antigens and demonstration of persistence of cellular antigens in apparent acellular scaffolds have brought into question the applicability of decellularization as the primary outcome measure in ECM scaffold production.^{98,185} Indeed, this concern has led to an increasing emphasis on assessment of both known (e.g., α -gal, MHC I) and unknown (e.g., total minor histocompatibility antigens) content of acellular ECM scaffolds.^{91,116,186–188}

Decellularization processes employ an array of mechanisms, including mechanical, osmotic, chemical, and/or enzymatic agents, which can be enhanced and/or combined to achieve better decellularization results.^{88,189–191} For instance, alterations to a detergent-based decellularization process by preventing vessel collapse through insertion of a rod in the lumen during decellularization process resulted in significant reduction of resultant scaffold immunogenic content.¹⁹² The resultant ECM scaffold demonstrated significant reduction of ECM scaffold immunogenic proteins, such as alpha smooth muscle actin, α -gal, and MHC-I complexes, as well as xenograft DNA content.¹⁹² This significant reduction of immunogenic proteins opens up the possibility of producing immunologically acceptable ECM scaffolds capable of minimizing immune rejection *in vivo*.

As noted previously the success of small diameter vessel ECM scaffolds depends not only on their ability to minimize adaptive immune rejection but also on retention of native structure–function properties to limit innate immune and nonimmune failure mechanisms. Unfortunately, further testing on rod-decellularized ECM scaffolds demonstrated that harsher decellularization methods had negative effects on the ECM structure and function.¹⁹³ Rod decellularization resulted in ECM scaffold distention, disruption of structural protein macromolecular structure and significantly reduced compliance. Consequently, despite the relatively encouraging effect of rod decellularization on resultant scaffold antigen content, disruption of the remaining ECM components induced by this approach has high risk of inducing innate immune and/or nonimmunogenic failure mechanisms (e.g., aneurysmal dilation).

AR has been proposed as a alternative tissue processing method, employing protein chemistry principles of differential solubilization to achieve stepwise removal of antigens with shared physiochemical properties (i.e., hydrophile solubilization through application of reducing conditions

and salting in, followed by lipophile solubilization through zwitterionic detergent use and nuclear antigen solubilization through nucleic acid digestion), showing promise in dramatically reducing resultant scaffold antigen content while maintaining native ECM structure–function properties.^{116,194}

Bovine pericardium (BP) treated with AR approach has yielded scaffolds with native ECM protein macromolecular structure. Encouragingly, AR methods significantly reduced α -gal, MHC I, and total minor histocompatibility antigens in BP, resulting in local graft-specific adaptive immune response, which was significantly less than that toward GTA-fixed BP.^{116,194} Furthermore, BP-AR scaffolds avoided proinflammatory innate immune response on *in vivo* implantation, ameliorating fibrosis and calcification associated with traditional decellularized BP.^{116,194}

Whether the result obtained for AR applied to BP would translate into small diameter vessel ECM scaffolds remains to be determined. Finally, it is worth pointing out the possibility of never reaching acceptable ECM scaffold immunogenicity through decellularization or AR approaches. Questions still remain concerning the relative immunogenicity of individual antigens, threshold level of elimination, which must be reached to achieve reduced adaptive immune response toward individual antigens and potential for tissue processing methods to expose cryptic antigens present in native tissue.

(ii) Altered mechanical properties: Use of xenogeneic tissue for small diameter vessel grafting is founded in the idea that native vessel contains the ideal mechanical properties for *in vivo* function. Therefore, any changes in scaffold mechanical properties risk disrupting the advantageous properties of the graft, while also increasing the risk of inducing nonimmune failure mechanisms such as calcification, aneurysm formation, and intimal hyperplasia. As a result, research studies have attempted to maintain native vascular tissue mechanical properties by either modifying the decellularization procedure to avoid disrupting mechanical properties of the resultant ECM scaffold, or increasing scaffold stability and associated mechanical properties after decellularization by addition of crosslinking and/or coating procedures.

Traditional decellularization methods (e.g., sodium dodecyl sulfate [SDS], trypsin, Triton X-100) have been reported to significantly disrupt ECM protein structure in a dose-dependent manner, which is reflected in changes to the scaffold mechanical properties.^{136,195–197} These alterations to the scaffold structural proteins and resultant mechanical properties can potentially lead to both immunogenic and nonimmunogenic failure mechanisms. Therefore, researchers had to modify decellularization procedures using non-denaturing chemicals in an attempt to reduce potential detrimental effects on the remaining structural ECM proteins. Modifications to the decellularization procedures often involve the use of novel compounds in combination with traditional decellularization steps.

Ammonium hydroxide or sodium hydroxide has been explored as decellularization agent.^{198,199} Decellularization using hydroxide components consist of disrupting the cells, followed by washes using mild soap or saline to remove cellular remnants. Decellularization using hydroxide components resulted in the production of ECM scaffolds with no histologically visible cellular components and with the capability of undergoing *in vitro* and *in vivo* recellularization.

This recellularization predominantly consisted of ECs and myofibroblasts.^{198,199} Unfortunately, partial degradation of collagen and elastin in hydroxide-decellularized vascular ECM scaffolds induced aberrant repopulating myofibroblast behavior and excessive production of ECM proteins. Similarly, alterations in ECM protein conformation induced macrophage infiltration and associated fibrosis, leading to failure in 50% to 60% of hydroxide-decellularized ECM scaffolds. In addition to degrading ECM proteins, hydroxide-based decellularization procedures have been reported to disrupt ECM scaffold mechanical properties, permeability, and conduit diameter compared with native tissue.^{198,199}

The disruptive effects on ECM scaffold structure/function relationships are not unexpected, given the fact that compounds like sodium hydroxide are commonly used to digest farm animal carcass. Although antigen content of hydroxide-decellularized tissues has not been assessed, the negative effects of this method on matrix structural elements make it unlikely to produce a viable solution regardless of adaptive immune response.

Other attempts at maintaining mechanical properties of the tissue have been to develop mechanical decellularization protocols that eliminate the need for harsh chemicals and allow tissue to maintain their original mechanical properties.^{70,73}

A common chemical-free decellularization protocol used in the field is the use of high hydrostatic pressure (HHP). HHP works by disrupting cell membranes through application of high pressure (980 MPa), followed by removing remnant cellular debris through washing.^{70,73,189} Throughout the literature, HHP-decellularized scaffolds have been consistently shown to result in nuclei-free scaffolds with significantly reduced levels of DNA content, while retaining the elastic lamina of the tissue.^{70,73,189} However, effect of HHP on collagen structure and retention has yielded inconsistent results, possibly due to differences in temperature at which pressurization profile was run, washing process, or both. Fortunately, this disparity in collagen structure and retention was not reflected in mechanical tests performed on HHP-decellularized ECM scaffolds. Burst pressure and suture retention tests of HHP-decellularized ECM scaffolds yielded results comparable with native tissue.^{70,73}

Despite alterations in collagen structure, results of *in vivo* implantation for HHP-decellularized vascular scaffolds are encouraging. HHP-decellularized vessels implanted into rat carotid arteries (ECM scaffold and allograft) and abdominal porcine aorta (allograft) remained patent for 2 weeks and 24 weeks, respectively.^{70,73,189} In addition, organized recellularization of the ECM scaffold was seen by ECs, alpha smooth muscle actin, and factor VIII-positive cells.⁷³ However, no information regarding the presence, or lack thereof, of innate or adaptive immune cells within the grafts was provided. The only information regarding the immunogenicity of HHP-decellularized ECM scaffolds is in the form of a qualitative assessment of inflammation.

Subcutaneously implanted HHP-decellularized ECM scaffolds demonstrated significant reduction in inflammation compared with native vessel at weeks 1 and 4 post-implantation.¹⁸⁹ However, adaptive immune response was not investigated in the reported model, and consequently inferences regarding the antigenicity of HHP-decellularized scaffolds are not available. Moreover, even though the

biggest advantage of HHP decellularization is the potential to maintain tissue mechanical properties, no article assessed these capabilities in a comprehensive manner, and questions regarding the immediate (e.g., compliance) and long-term (e.g., cyclic strain) mechanical effect of collagen disruption in such scaffolds remain. This lack of testing makes it difficult to conclude HHP decellularization is in fact better at maintaining the tissue original mechanical properties than detergent-based decellularization approaches.

Other researchers have focused on improving the mechanical properties of decellularized vascular ECM scaffolds by combining the decellularization process with terminal crosslinking or reinforcement by synthetic polymers.^{75,200,201} As mentioned previously, crosslinking chemically stabilizes tissue, which has potential to improve mechanical properties, thereby reducing risk of aneurysmal dilation. Similarly, using synthetic material to reinforce the small diameter vessel xenograft has the potential to improve mechanical properties.^{75,200,201}

Recently, pentagalloyl glucose (PGG) and poly(ethylene glycol) diglycidyl (PDGE) have been tested as crosslinking agents, while electrospun polycaprolactone (PCL) has been tested as a synthetic reinforcement.^{75,200,201} PGG-crosslinked scaffolds are produced by selectively removing cells and collagen using sodium hydroxide decellularization methods. Crosslinking is then achieved by soaking in PGG, which works as an elastin stabilizing polyphenolic tannin. PCL-reinforced scaffolds are produced by osmotic shock, enzyme digestion, and mechanical processes and reinforced by electrospinning PCL around the scaffold, producing a bilayered structure.

PGG-crosslinked and PCL-reinforced scaffolds demonstrated complete acellularity and significant reduction in DNA content.^{75,201} However, scaffolds were shown to have altered collagen and elastin fibers.^{75,200,201} Alterations to the ECM structural proteins seem to be compensated by the PGG crosslinking and PCL reinforcement, as the majority of the assessed mechanical properties were not significantly different than those of native tissue.^{75,201} This finding suggests that PGG crosslinking and PCL reinforcement can restore the mechanical properties of ECM scaffolds, which are damaged due to the decellularization process. However, compliance of PGG-crosslinked decellularized scaffolds was 50% lower than that of native.⁷⁵ PGG-heparin and PCL-heparin bound scaffolds were tested *in vivo*, for a maximum of 8 and 6 weeks, respectively, and resulted in 100% patency.^{75,201} The ECM scaffold structure remained intact and underwent high cell infiltration. Unfortunately, a portion of the infiltrating cells consisted of foreign body giant cells and lymphocytes, known to cause proinflammatory foreign body type immune responses.^{75,201} Other infiltrating cells consisted of M1 and M2 macrophages; however, no information regarding their relative proportions was provided.⁷⁵

Information about the relative proportions of M1 and M2 macrophages would have been useful to determine the possible fate of the ECM scaffolds as the M1/M2 macrophage ratio can potentially discriminate between a proinflammatory immune response (M1-like) or anti-inflammatory immune response (M2-like) toward the ECM scaffold.⁸⁷ Consequently, although crosslinking or reinforcement may restore mechanical properties of ECM scaffolds in which the decellularization process resulted in disruption of ECM structural proteins, it

TABLE 2. SUMMARY OF COATING COMPOUNDS UTILIZED IN REVIEWED MANUSCRIPTS TO MODULATE CELL BEHAVIOR ON EXTRACELLULAR MATRIX SCAFFOLDS *IN VITRO* AND THE REPORTED EFFECTS ON CELLULAR BEHAVIOR ANALYZED ON EACH MANUSCRIPT

	<i>Brain-derived neurotrophic factors (BDNF)</i> ⁷⁶	<i>Integrin α4β1 ligand peptide (REDV)</i> ⁷⁸	<i>Vascular endothelial growth factor (VEGF)</i> ²⁰⁴	<i>Polydopamine (pDA)</i> ²⁰⁷	<i>Chitosan/beta-GP gel</i> ²⁰⁶
Adhesion	X	x	x	x	N/A
Proliferation	N/A	N/A	N/A	x	N/A
Differentiation	N/A	N/A	N/A	x	N/A
Migration	X	x	x	N/A	X
Survival	X	N/A	x	N/A	N/A
Type of cell used	Endothelial progenitor cells	Human umbilical vein endothelial cells	Human umbilical vein endothelial cells	Endothelial progenitor cells	Mesenchymal stem cells

N/A means not assessed.

may be insufficient to overcome recipient graft-specific immune responses toward such non-native structural elements.

(iii) Improper recellularization: The capability of xenogeneic ECM scaffolds to elicit cell migration, proliferation, and infiltration is key to recapitulate native vessel cellular organization and associated long-term graft patency. Furthermore, as previously discussed rapid cellular repopulation with a healthy quiescent EC monolayer is critical to avoid thrombosis and limit potential for intimal hyperplasia after *in vivo* implantation. Consequently, retention of native vascular ECM proteins is likely to be crucial for proper recellularization.²⁰² Unfortunately, a broad review into different decellularization methods has reported the inability for any process to retain native ECM protein structure, composition, and organization.¹⁹¹ In particular, disruption of delicate endothelial basement membrane components (e.g., Col IV, Laminin) has been widely reported with decellularization approaches, regardless of specific chemicals employed.¹⁹¹

In nonvascular scaffolds, preserved basement membrane components have been demonstrated to enhance repopulating cell adhesion, proliferation, and migration compared with nonbasement membrane surfaces.^{202,203} However, the extent to which preservation of luminal basement membrane components in vascular ECM scaffolds may be capable of modulating repopulating ECs behavior remains unknown. The importance of promoting rapid quiescent endothelial monolayer formation has driven investigation of numerous

approaches to ameliorate the effect of alterations in ECM protein properties, thereby enhancing endothelialization of vascular ECM scaffolds.

The most common approach, identified in the articles reviewed for this article, to avoid thrombosis due to lack of an EC monolayer in ECM scaffolds has been to modify the luminal scaffold surface using a variety of coatings.^{69,71,74,76,78,204–208} Coatings have generally been designed to either target enhanced cell repopulation or serve as an antithrombotic barrier, with some researchers using combinations of both approaches in an attempt to enhance overall *in vivo* outcome. Proteins such as growth factors and integrins have been utilized as coating agents to stimulate cell repopulation.^{69,76,78,204–207} The most widely utilized barrier compound is heparin.^{69,71,74,204,208} One final approach has employed thrombospondin-2 knockout (TSP2 KO) ECM to examine the effect of elimination of pro-thrombotic molecules from the resultant scaffold.⁷²

The effect of scaffold modification by coating has been reported to be generally positive, despite the major differences in the mechanism of action of the differing coating molecules. Vascular ECM scaffolds coated with barrier compounds underwent an increase in *in vitro* antithrombogenic activity, cell adhesion, and fibrin deposition.^{69,72,74,208} Similarly, barrier-only coatings generally resulted in *in vivo* decreased platelet aggregation, calcification, and fibrin deposition and increased cell adhesion.^{71,72,74} *In vitro* cell studies using compounds that modulate cell behavior

TABLE 3. SUMMARY OF *IN VIVO* EXPERIMENTS OF SMALL DIAMETER EXTRACELLULAR MATRIX SCAFFOLDS COATED WITH CELL MODULATING COMPOUNDS REPORTED IN REVIEWED ARTICLES

	<i>Brain-derived neurotrophic factors (BDNF)</i> ⁷⁶	<i>Integrin α4β1 ligand peptide (REDV)</i> ⁷⁸	<i>Vascular endothelial growth factor (VEGF)</i> ²⁰⁴	<i>VEGF conjugated to a temperature-sensitive aliphatic polyester hydrogel (VEGF-HG)</i> ²⁰⁵
Type	Allograft	Xenograft	Xenograft	Allograft
Heparin coating	No	No	Yes	No
Transplant species/site	Rat/carotid artery	Mini pig/femoral artery	Sheep/carotid artery (~4 cm in length)	Rat/infrarenal aorta (~1–2 mm diameter)
Time	2 months	3 weeks	3 months	8 weeks
Patency rate (%)	90	83	83	100

demonstrated proof-of-principle for the ability of growth factors and cell adhesion molecules to modulate cell behavior on ECM scaffolds. Depending on the specific molecule applied to the ECM scaffold cell adhesion, proliferation, differentiation, migration, and/or survival can be induced (Table 2).^{76,78,204,206,207} Studies that include the use of coated small diameter vessel ECM scaffolds *in vivo* reported high patency rates (patency rate) over periods of 3 (83%—xenograft) and 8 (100%—allograft) weeks and 2 (90%—allograft) and 3 (83%—xenograft) months (Table 3).^{76,78,204,205} In all cases, scaffolds demonstrated robust smooth muscle and EC recellularization.^{76,78,204,205} Finally, the combination of barrier and cell modulating compounds resulted in reduced production of blood coagulation factor *in vitro*, as well as reduced platelet accumulation and fibrin deposition *in vivo*.⁶⁹

High *in vivo* patency rates and high cell presence suggest that scaffold coating may serve as a solution to achieving rapid EC repopulation of ECM scaffolds. However caution must be exercised before use of coatings in the clinical setting due to the potential for severe side effects when utilizing such modifications of native scaffold biology. For example, integrin $\alpha 4 \beta 1$ ligand has been utilized as a coating agent due to its role in promoting cell adhesion.⁷⁸ However, the possibility of enhancing graft-specific immune response toward such grafts should be considered as a potential complication. Although $\alpha 4 \beta 1$ integrin plays a role in endothelial and smooth muscle cell adhesion, it is also highly expressed on T cells, B cells, monocytes, and natural killer cells among other inflammatory cells.^{209,210} Enhanced recruitment of such adaptive and innate immune cells has potential to result in both short- and long-term graft dysfunction as previously discussed.

Vascular endothelial growth factor coating has been explored for its important role in angiogenesis, EC migration, and proliferation.^{204,205} However, it has also been demonstrated that uncontrolled exposure to VEGF can lead to abnormal cell proliferation and migration, leading to intimal hyperplasia and potential for tumor formation.^{205,211,212} Consequently, although coatings designed to enhance cell recruitment show promise in enhancing endothelialization of vascular ECM scaffolds, future research into coating approaches that are capable of recruiting specific target cells is needed to avoid recruitment and activation of potentially detrimental cellular subtypes.

In vitro recellularization of vascular ECM scaffolds has also been investigated as a method to provide an immediate endothelial barrier upon implantation.^{62,67,77} Furthermore,

in vitro cell seeding provides researchers flexibility to equip scaffolds with differing cell types (e.g., ECs, smooth muscle cells) to more directly recapitulate native vessel function before *in vivo* implantation. Regardless of the specific EC type used, *in vivo* results of scaffold seeding are largely positive compared with unseeded scaffolds.

Cell seeding resulted in scaffolds with high patency rates (patency rate) over periods of 3 months (100%—allograft, 95%—allograft) and 5 months (allograft), with reduced levels of thrombus formation compared with unseeded allograft controls (Table 4).^{62,67,77} However, the experimental design for these *in vivo* studies makes it difficult to attribute these two major accomplishments to cell seeding alone. First, the authors claim that cell seeding is efficient at reducing thrombosis, as there is a consistent decrease of thrombi. However, heparin coating was also employed in the noncontrol scaffolds, making it impossible to separate the antithrombotic effect of the seeded cells from that of the heparin coating. In addition, the fact that all *in vivo* studies were allotransplants, instead of xenotransplants, prevents interpretation of possible adaptive immune responses on graft patency rates.

Abundant research of engineered vascular grafts by cell seeding has demonstrated numerous positive results, particularly when seeded with stem cells or stem cell-derived cells. This topic is further reviewed elsewhere.²¹³

Other researchers have moved away from trying to solve the problem of cell repopulation by modifying the ECM scaffold preimplantation with the goal of positively influencing host remodeling of the scaffolds, postimplantation. This has been attempted using systemic postoperative injections of granulocyte colony-stimulating factor (G-CSF).^{68,214} G-CSF is a glycoprotein known to stimulate the production and release of stem cells and granulocytes by the bone marrow (BM).^{215,216} Evidence suggests that the stem cells produced by G-CSF stimulated-BM target denuded vessel walls, enhancing the recellularization process and reducing intimal hyperplasia, ultimately restoring vascular activity.^{86,217–220} Unfortunately, consistent protocols for administration of G-CSF have yet to be determined. Consequently, comparison between studies is currently challenging due to differences in the specific protocols employed. G-CSF is consistently administered subcutaneously; however, dosage, length of administration, and initial day of injections vary between studies.^{68,214}

Regardless of differences in administration of C-GSF, recent studies reported high patency rates (patency rate)

TABLE 4. SUMMARY OF REPORTED *IN VIVO* EXPERIMENTS OF SMALL DIAMETER EXTRACELLULAR MATRIX SCAFFOLDS SEEDED WITH CELLS BEFORE IMPLANTATION

	Microvascular endothelial cells ⁶⁷	Endothelial progenitor cells ⁷⁷	Endothelial-like and smooth muscle-like cells derived from mesenchymal stem cells ⁶²
Type	Allograft	Allograft	Allograft
Time	3 months	3 months	5 months
Transplant species/Site	Rat/abdominal aorta	Dog/carotid artery	Sheep/carotid artery
Patency rate (%)	100	95	Dns

Although *in vitro* studies for all three manuscripts focused on development of xenogeneic scaffolds, *in vivo* experiments utilized allograft versions of the resultant scaffolds.

over periods of 8 weeks (100%—allograft) and 6 months (95%—allograft), as well as decreased intimal hyperplasia of decellularized scaffolds when compared with controls.^{68,214} However, it is difficult to conclude the overall effect of G-CSF on scaffold recellularization, as the remainder of the results are contradictory between manuscripts.^{68,214} Min Zhou *et al.* reported treatment with G-CSF resulted in increased ECs repopulation, while Joonkyu Kang *et al.* found no difference when compared with untreated animals. The different results can potentially be attributed to the differences in experimental setup, as the G-CSF administration protocol and study duration differed between the two reports. Regardless of the reasons for such disparate results, more data are required to determine the potential of G-CSF administration to enhance vascular ECM scaffold recellularization *in vivo*. Aside from contradicting results, injected G-CSF has the potential to become problematic for scaffold *in vivo* patency, since it also stimulates the BM to produce and release granulocytes, which has potential to enhance proinflammatory immune response toward decellularized scaffolds.^{99,137}

Conclusion

Xenogeneic small diameter vascular ECM scaffolds are an attractive solution to overcome the deficiencies of autologous vessel use in CABG. Indeed, the promise of ECM scaffolds in vascular replacement has led to an intense research effort toward this goal. Previous research has highlighted and defined the challenges to development of successful small diameter vascular ECM scaffolds. Despite the number, complexity, unforgiving nature of the failure mechanisms identified for small diameter vascular ECM scaffolds, progress has been made. As detailed in this article, approaches have been developed to overcome each of the primary failure mechanisms for such scaffolds. Efforts dedicated to solve the limitations of small diameter vessel ECM scaffolds have researched a vast array of modifications, conditionings, and chemical changes to improve upon the *in vivo* outcome of the scaffolds. Different techniques have resulted in different degrees of success, which have all contributed valuable information to the field.

Conclusively and extensively reported through this review and acknowledged in most relevant papers, the success of a small diameter vessel ECM scaffold is completely dependent on eliminating the three main conditions for scaffold failure: (i) proinflammatory immune response, (ii) altered mechanical properties, and (iii) improper recellularization. Contradictorily, most of the recent work done on small diameter vessel ECM scaffolds focus on ameliorating a single condition at the time, which, in some cases, results in improving a condition at the expense of another one, making it nearly impossible to succeed at developing a patient-ready small diameter vessel ECM scaffold. Therefore, determining the combination of factors required to overcome all failure mechanisms in a single graft remains elusive. Ensuring that new approaches are focused on overcoming the primary failure mechanisms identified for vascular ECM scaffolds and comprehensive assessment of all of these factors is undertaken using *in vivo* models is critical for further progress in the field.

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