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Emt Driven By Tgf-B Is A Significant Mediator Of Stenosis In Tissue Engineered Vascular Grafts

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EMT driven by TGF-B is a significant mediator of stenosis in tissue engineered vascular grafts

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Abstract

The development of an autologous tissue engineered vascular graft (TEVG) holds great promise for improving outcomes in congenital heart surgery. The first clinical trial showed that this approach is safe and effective but that the primary mode of failure is stenosis. In a C57BL/6 mouse model of unseeded TEVGs implanted as inferior vena cava interposition grafts, neovessels form in 2 weeks and patent grafts show endothelial and smooth muscle cell (SMC) layers, but occluded grafts show accumulation of SMCs. This suggests stenosis results from abnormal SMC in-growth in the neointima. Studies on fibrosis have shown that resident endothelial cells (EC) contribute to fibroblast accumulation through endothelial-mesenchymal transition (EMT). We utilized transgenic EC lineage-tracing mouse models to track the occurrence of EMT in our TEVG and found an increased percentage of cells co-expressing LacZ and smooth muscle actin in occluded grafts, suggesting that EMT contributes to occlusion in our TEVG. We hypothesized that ECs in our TEVG undergo EMT driven by TGF-B to contribute to stenosis. Immunohistochemistry and qRT-PCR showed higher expression of TGF-B in occluded compared to patent grafts. In vivo expression of a soluble FGF trap virus to increase TGF-B signaling and thus increase EMT was found to result in a significantly increased stenosis rate in our TEVG. We next modulated this pathway by intraperitoneal administration of a small molecule inhibitor of TGF-B receptor type 1 (SB431542). Grafts from treated mice had significantly increased patency rates and internal diameters at 2 weeks compared to controls while maintaining normal neovessel architecture. We then designed a novel local delivery system for this TGF-B R1 inhibitor in our grafts and showed that local drug delivery inhibits stenosis without cell seeding and maintains normal neovessel formation. These results suggest that EMT under the control of TGF-B is a significant mediator of stenosis and that modulation of this pathway by local drug delivery might be useful in next generation TEVGs.

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List of Abbreviations

BM-MNC: Bone marrow mononuclear cells

CHD: Congenital Heart Disease

EC: Endothelial Cell

EC TCPC: Extra Cardiac Total Cavopulmonary Connection

ECM: Extracellular matrix

EMT: Endothelial-mesenchymal transition

FGF: Fibroblast Growth Factor

H&E: Hematoxylin and eosin stain

IHC: Immunohistochemistry

IVC: Inferior vena cava

qRT-PCR: Quantitative Real Time Polymerase Chain Reaction

SMA: Smooth muscle actin

SMC: Smooth muscle cell

TEVG: Tissue engineered vascular graft

TGF-B: Transforming Growth Factor B

TGFBR1: TGF-B Receptor Type 1

vWF: Von Willebrand Factor

Introduction

Clinical Background

Despite major advances in medical and surgical treatment, congenital heart disease (CHD) remains the leading cause of death due to congenital anomalies in the newborn period [1]. Single ventricle anomalies make up one of the largest groups of cardiac anomalies resulting in life-threatening diseases. These include diseases such as tricuspid atresia, pulmonary atresia, and hypoplastic left heart syndrome, in which only one ventricle is of adequate functional size. These anomalies result in mixing of the deoxygenated pulmonary circulation and the oxygenated systemic circulation, causing chronic hypoxia and cyanosis. This mixed circulation can cause volume overload that can lead to heart failure. Untreated single ventricle anomalies are associated with up to 70% mortality during the first year of life [2]. The treatment of choice for this CHD is surgical reconstruction, the goal of which is to separate the pulmonary circulation from the systemic circulation, preventing cyanosis, volume overload, and heart failure [3, 4]. This is accomplished through a series of staged procedures referred to as the modified Fontan operation with extra cardiac total cavopulmonary connection (EC TCPC). This operation has considerably improved long-term survival but is considered only a palliative procedure with significant morbidity and mortality [3, 4].

An important cause of complications in EC TCPC is the conduit used to connect the inferior vena cava (IVC) to the pulmonary artery [5]. Much of the late morbidity is attributed to problems with conduit use [6] and while as many as 10,000 children undergo such reconstructive cardiothoracic operations each year, it is widely accepted that the ideal conduit has not yet been developed [7-9]. Data describing long-term graft failure

rates for conduits used for EC TCPC is limited but long-term data for similar congenital heart conduit operations suggest outcomes are poor [10]. Late problems include conduit degeneration with progressive obstruction and susceptibility to infection. Synthetic conduits lack growth potential, necessitating re-operation when a pediatric patient outgrows the graft. Synthetic conduits are also a significant cause of thromboembolic complication due to the area of synthetic material in contact with blood causing activation of the coagulation cascade [11]. It is assumed that all such conduits eventually need to be replaced. Re-operation is associated with significant morbidity and early post-operative mortality rates as high as 5% [10]. Long-term graft failure rates have been reported at 70-100% at 10-15 years [12, 13]. The best results have been obtained when autologous tissue was used for the conduit with long-term patency rates of over 80% [14]. Autografts, conduits created from an individual's own tissue, have better long-term effectiveness than any synthetic or biological conduit currently available but these are limited in supply, suggesting the need for an alternate approach [10, 13-15].

Tissue Engineering Approach

Tissue engineering offers a strategy for constructing autologous grafts and thereby increasing the pool of potential autografts for use as conduits [16]. Using the classical tissue-engineering paradigm, autologous cells can be seeded onto a biodegradable tubular scaffold, which provides sites for cell attachment and space for neotissue formation [17]. As the neotissue forms, the scaffold degrades creating a purely biological graft. The resulting neotissue can thus function as a vascular graft in cardiothoracic operations [18].

Extensive large animal studies have demonstrated the feasibility of using tissue-engineering methodology to construct conduits for use as large grafts [18-21].

Clinical Trial

Based on the success of animal studies, Shinoka performed a pilot clinical study in Japan in 2001 to evaluate the feasibility and safety of using a tissue engineered vascular graft (TEVG) as a conduit for EC TCPC in patients with single ventricle cardiac anomalies [22-24]. Twenty-five TEVGs seeded with autologous bone marrow mononuclear cells (BM-MNC) were implanted with follow-up out through seven years [19, 25]. At the most recent follow-up, the tissue engineered vascular grafts were shown to function well without evidence of graft failure. No graft had to be replaced and there was no graft related mortality. An additional advantage of this technology is almost eliminating the need for antiplatelet, antigoagulant, and immunosuppressive therapy. All patients had both antiplatelet and anticoagulant medications discontinued by 6 months postoperatively and 40% of patients remained free of any daily medications long term in stark contrast to the lifetime need for anticoagulation with the use of synthetic grafts [22]. Long-term follow-up, however, revealed graft stenosis in 16% of patients (Table 1). Stenosis in these patients was frequently asymptomatic and all were successfully treated with angioplasty and stenting. In addition, serial imaging demonstrated the growth potential of these grafts, an element that is extremely important in the pediatric population (Figure 1). These data support the overall feasibility and safety of using vascular tissue-engineering technology in the pediatric clinical setting [22].

Table 1: Late term status after TEVG implantation in clinical trial

Patient	Age at Operation (Years)	Patient Status	Graft Status	Graft Patency	Graft Related Complications
1	2	alive	intact	patent	none
2	1	alive	intact	patent	none
3	7	alive	intact	patent	stenosis
4	21	alive	intact	patent	none
5	4	alive	intact	patent	none
6	12	alive	intact	patent	none
7	17	alive	intact	patent	none
8	19	dead	intact	patent	none
9	3	alive	intact	patent	stenosis
10	2	dead	intact	patent	none
11	13	alive	intact	patent	stenosis
12	2	dead	intact	patent	none
13	2	alive	intact	patent	thrombosis
14	2	alive	intact	patent	none
15	2	alive	intact	patent	none
16	2	alive	intact	patent	none
17	24	alive	intact	patent	none
18	1	alive	intact	patent	stenosis
19	11	alive	intact	patent	none
20	2	alive	intact	patent	none
21	3	alive	intact	patent	none
22	4	alive	intact	patent	none
23	4	alive	intact	patent	none
24	13	alive	intact	patent	none
25	2	dead	intact	patent	none

Table 1: Late term status after TEVG implantation in clinical trial. Most recent follow-up at mean of 5.8 years showed no graft-related mortality and no evidence of aneurysm formation, graft rupture, or ectopic calcification. 4 out of 25 patents developed asymptomatic stenosis that was picked up on routine serial imaging and were successfully treated with angioplasty. All implanted TEVG are currently intact and patent. (Adapted from Hibino (2010) [22]).

Figure 1: Growth potential of TEVG in clinical trial

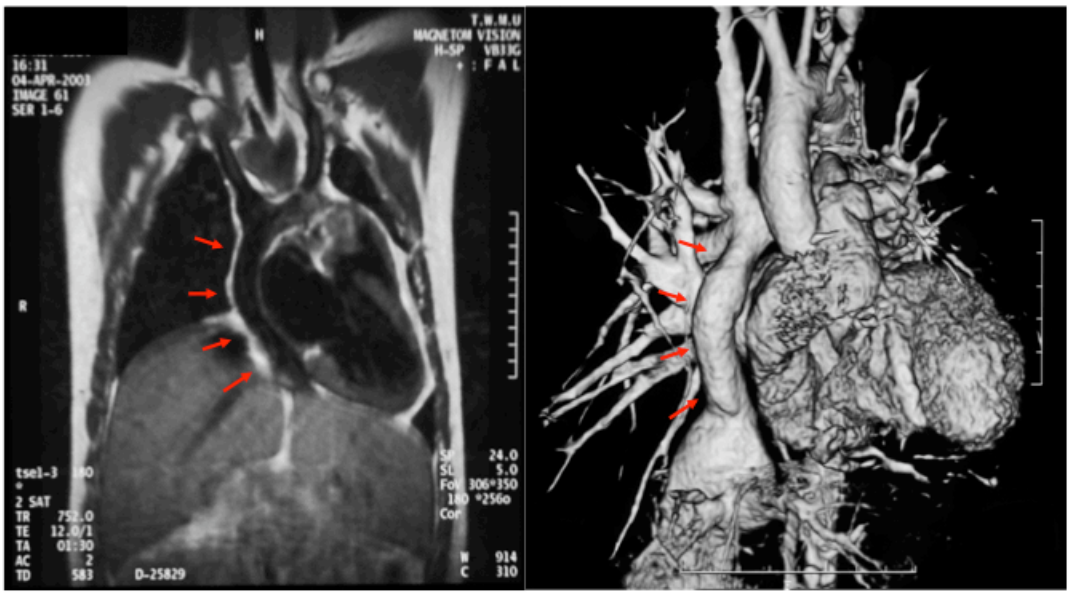

Figure 1: Growth potential of TEVG in clinical trial. A. Magnetic resonance image (MRI) 9 months following implantation of EC TCPC graft. B. 3-D computed tomography angiogram (CTA) of graft one year after implantation. Red arrows indicate location of tissue engineered vascular graft. (Adapted from Shinoka (2008) [26]).

Complications arising from the use of currently available synthetic vascular grafts are a leading cause of morbidity and mortality after congenital heart surgery [5]. The lack of growth potential of synthetic conduits is problematic. Use of over-sized grafts in an attempt to avoid outgrowing a conduit is widely practiced, but graft over-sizing has an increased risk of complications [27]. Delaying surgery to minimize re-operations can lead to cardiac dysfunction or heart failure due to prolonged exposure to volume overload and chronic hypoxia [11]. The development of a vascular graft with growth potential would eliminate this problem. Review of the data suggest that the safety and efficacy of the use of TEVGs in congenital heart surgery is excellent, but mechanisms underlying the process of neovessel formation that lead to TEVG failure have remained incompletely

understood. Exploring these processes is essential to create an improved tissue engineered vascular conduit. Also, as noted at long-term follow-up it was found that the primary mode of failure for TEVG is stenosis [18-22, 25]. Identification of the mediators of stenosis in TEVG and determination of the mechanisms underlying neovessel formation would identify targets and potential strategies for preventing stenosis and thereby enable the rational design of improved TEVG.

Mechanisms of Neotissue Formation

Neotissue Growth

In order to better study the mechanisms of TEVG formation and stenosis in vivo, mouse models have been developed to recapitulate the results of the human trial (Figure 2). This approach includes a method for constructing sub-1mm tubular scaffolds similar to the scaffold used in the clinical trial [28]. These scaffolds can be seeded with cells to create TEVG. Use of immunodeficient SCID-beige mice has enabled transplantation of human cells or cells from strains of transgenic mice without the need for immunosuppression. This has proven to be an excellent model for evaluating TEVG [29, 30]. In an initial pilot study TEVG were implanted as infrarenal IVC interposition grafts and observed over a six-month time course to determine the effect of human BM-MNC seeding on neovessel formation. The seeded TEVG functioned well and had better long-term graft patency and less stenosis than the unseeded scaffolds [31]. Quantitative morphometric analysis demonstrated that unseeded TEVG had significantly increased wall thickness and luminal narrowing compared to seeded TEVG. Further analysis revealed that the primary mode of failure is stenosis characterized by graft wall

thickening and progressive luminal narrowing, which ultimately leads to luminal obliteration and vessel occlusion by inward remodeling. Cell seeding appears to inhibit inward remodeling and promote outward remodeling in neovessel formation [31].

Figure 2: TEVG in a mouse model

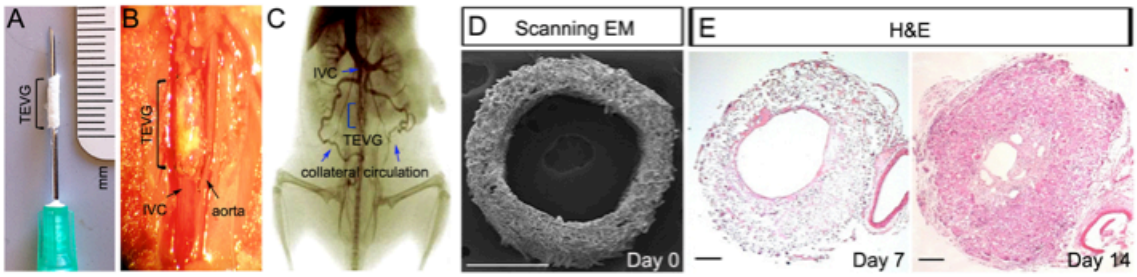

Figure 2: TEVG in a mouse model. A. Gross image of PGA-P(CL/LA) TEVG before surgical implantation. Scale bar: 1 mm. B. Intraoperative photograph of an inferior vena cava (IVC) interposition graft in C57BL/6 mouse. C. Venous phase contrast CT of occluded TEVG in CB17 mouse. D. Scanning electron microscope image of PGA-P(CL/LA) TEVG. Scale bar: 500 μm. E. Hematoxylin and eosin (H&E) stain of TEVG at day 7 and day 14. Scale bar: 200 μm.

A series of time course experiments using ovine and canine models demonstrated the stepwise morphologic changes that occur when a seeded scaffold is implanted as a vascular interposition graft [19-21, 32]. The process begins with a host-derived inflammatory response followed by formation of a monolayer of endothelial cells lining concentric layers of smooth muscle that develop on the luminal surface of the scaffold. As the scaffold degrades, the cells produce an extracellular matrix rich in collagen, elastin, and glycosaminoglycans, resulting in the formation of a neovessel with biomechanical properties similar to native blood vessel complete with intimal, medial, and adventitial layers that histologically resemble native vessel. The vascular neotissue

shows evidence of normal growth and development including increase in size proportional to the surrounding native tissue and expression of Ephrin B4, the molecular determinant of veins, when implanted as an IVC graft [32].

Neotissue Remodeling

Extensive histological and immunohistochemical (IHC) characterization has been performed to show the changes in TEVG over time in a murine model and these have documented the natural history of neovessel formation, the process of developing from a biodegradable tubular scaffold seeded with BM-MNC into a vascular conduit that resembles and functions as a native blood vessel. Six-months after implantation, the resulting neotissue possesses an internal monolayer of endothelial cells surrounded by inner smooth muscle layers, and an organized extracellular matrix. Some groups have hypothesized that stem cells within the bone marrow cell population differentiate into the cells of the neotissue [33], but characterizing the human BM-MNC population, however, revealed very few endothelial cells, smooth muscle cells and vascular progenitor cells and therefore it seemed that the seeded cells were unlikely to be the ultimate source of the vascular neotissue. This hypothesis is not consistent with classic tissue engineering theory which views the seeded cells as building blocks of neotissue, but instead supports a regenerative medicine paradigm in which the seeded scaffold is used to augment the body's own reparative mechanisms to "regenerate" missing tissue. To test this hypothesis, species-specific IHC stains were used to determine the fate of the seeded human BM-MNC in the mouse host. Results of these studies revealed that seeded cells were replaced by host cells one to three-weeks after implantation. These findings were

confirmed using human specific GAPDH RNA detection via qRT-PCR, confirming the presence of human RNA on the TEVG prior to implantation followed by a dramatic decrease such that no human RNA could be found by post-operative day 7 [31].

Based on these preliminary studies it has been hypothesized that seeded cells exert their effect via a paracrine mechanism by releasing chemokines that recruit host cells to the scaffold. These host cells are then critical for vascular neotissue formation and promote outward remodeling to maintain graft patency. IHC characterization demonstrated that the TEVG were initially infiltrated by host-derived monocytes and macrophages. Based on quantitative IHC data a correlation was noted between degree of early inflammatory response and graft patency. Specifically, the seeded grafts had significantly more macrophages in the early period compared to unseeded vascular grafts, suggesting that macrophage recruitment may be important in the process of promoting outward remodeling during neovessel formation. IL-1B and MCP-1 were found to be produced in abundant quantity. Studies of TEVG seeded with BM-MNC from MCP-1 knockout mice or wild-type implanted into a SCID-beige vascular interposition graft model revealed that TEVG seeded with MCP-1 knockout BM-MNC developed significantly more wall thickening and luminal narrowing, suggesting that MCP-1 plays a critical role in inducing outward remodeling. Alginate microspheres were created and incorporated into the wall of the scaffold to provide controlled release of MCP-1. A study using this scaffold showed that an MCP-1 eluting scaffold can inhibit stenosis in the absence of BM-MNC seeding. These studies suggest that BM-MNC seeded scaffolds transform into functional vessels by means of an inflammation-mediated process of vascular remodeling (Figure 3) [31].

Figure 3: TEVG remodeling in a mouse model

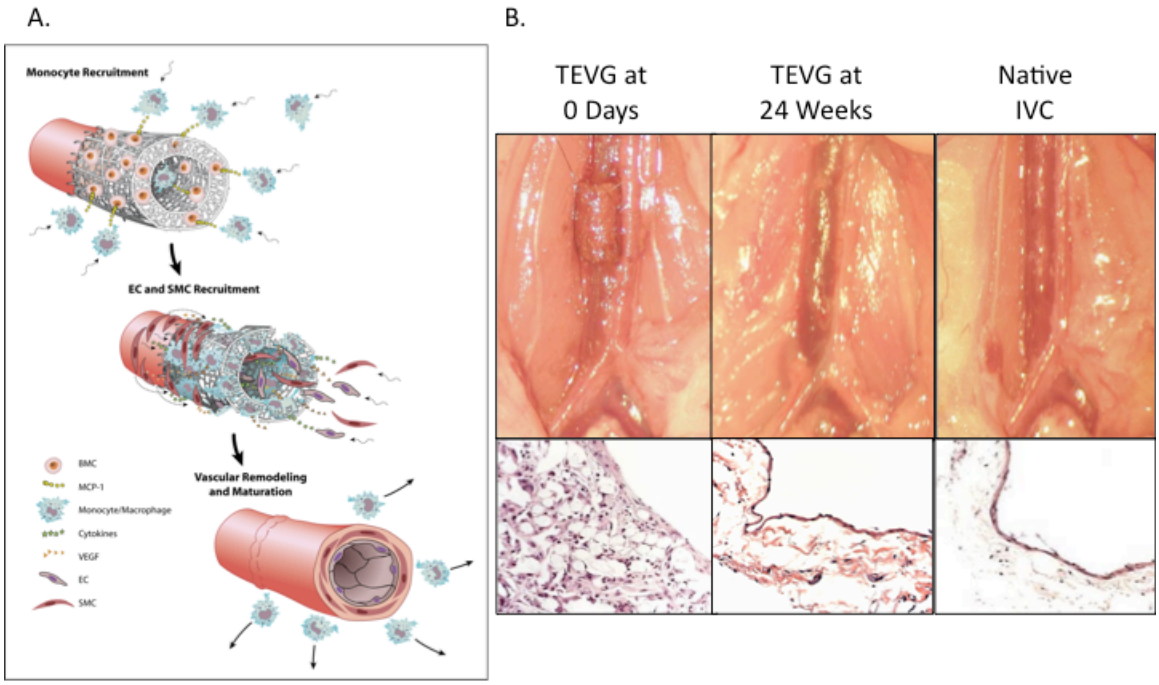

Figure 3: TEVG remodeling in a mouse model. A. Inflammation-mediated process of graft remodeling. Seeded BM-MNC attach to the scaffold and release cytokines. MCP-1 recruits host monocytes which infiltrate the scaffold and begin to direct vascular neotissue formation, ultimately resulting in the formation of neovessels composed of a concentric layers of smooth muscle cells recruited from the neighboring native vessel wall embedded in an extracellular matrix with a monolayer of endothelial cells lining the luminal surface. B. TEVG gross and microscopic morphology changes over time and ultimately resembles the native IVC with a smooth muscle cell layer lined by an endothelial cell layer as shown in gross images and hematoxylin and eosin stained section slides. (Adapted from Roh (2010) [31]).

According to this model, the seeded BM-MNC attach to the scaffold and begin to release MCP-1. Once implanted as an IVC interposition graft, MCP-1 recruits host monocytes which infiltrate the scaffold and begin to direct or participate in vascular neotissue formation, ultimately resulting in the formation of neovessels composed of concentric layers of smooth muscle cells recruited from the neighboring native vessel wall embedded in an extracellular matrix with a monolayer of endothelial cells lining the

luminal surface [31]. Recent studies used composite grafts consisting of male vessel segments implanted into female mice and wildtype mice given GFP bone marrow transplants to determine the source of neotissue cells. These studies showed that the cells of the neovessel do not derive from the bone marrow or the seeded cells but actually arise as a result of migration from the adjacent vessel segment as an augmented regenerative response [34].

Clinical Trial at Yale School of Medicine

It will be important to have a deeper understanding of the mechanisms of neotissue formation and stenosis for an FDA approved clinical trial that has been initiated at Yale School of Medicine to investigate the use of TEVG in pediatric patients [26, 35]. All elements of the process of TEVG development need to be considered in this context including scaffold materials, cells for seeding grafts, seeding techniques, patient selection, and imaging advances.

Scaffold Materials

Scaffold materials must be biodegradable and non-immunogenic. They must provide space for cell attachment while allowing for appropriate structural integrity until neotissue can form. Standard approaches involve the use of polymers of polyglycolic acid (PGA), polylactic acid (PLA), and poly e-caprolactone (PCL) in varying concentrations to meet the compliance specifications of the vascular system into which the graft is being introduced [36, 37]. Electrospinning is a newer approach for creating vascular graft scaffolds that can be made with finely tuned biomechanical specifications

[38]. Other groups have pioneered the use of decellularized biologic materials including human and porcine vessels [39]. Additional novel approaches involve the use of human umbilical vein as a living scaffold and grafts made using sheets of a patient's own fibroblasts [40-42].

Cells for Seeding

Many cell types have been considered as possibilities for seeding vascular grafts [43, 44]. Some groups have investigated the use of endothelial cells and smooth muscle cells for seeding but these approaches require much longer incubation times, presenting additional risk of contamination along with a slower process to make TEVGs ready for implantation. Recent investigation has focused on shortening the time required for this approach, including the use of novel flow chambers and other bioreactors [44].

Bone marrow mononuclear cells have been found to be useful for seeding as they are readily available from patients by means of bone marrow aspiration and show the most promise in inducing proper neotissue formation [45]. There are several different approaches for purifying mononuclear cells from the bone marrow. The traditional approach has involved Ficoll centrifugal separation based on cell mass, but this takes several hours. A newer approach involves using a specially designed filter to separate out cells of a particular size [46]. Alternative methods need to optimize speed and specificity for the cells of interest, while maintaining sterility and cell viability.

Alternative cells that might allow for even more effective cell seeding could include embryonic stem (ES) cells or induced pluripotent stem (iPS) cells, a newer autologous approach to developing pluripotent cells [47-49]. All pluripotent cells present

the risk of teratoma formation and so more investigation is needed into the use of these cell types for the seeding of TEVGs. It is yet to be seen whether an optimal approach would involve seeding with undifferentiated ES or iPS cells or rather using these cells derived from a patient to make a differentiated cell line of smooth muscle and/or endothelial cells for the seeding of vascular grafts [50].

Seeding Techniques

The traditional approach to placing cells on a scaffold for TEVG creation is static cell seeding, in which the patient's cells are pipetted directly onto a graft before being given several hours to attach. There are a number of recognized shortcomings of the static seeding method, including lower final seeding efficiency and inter-operator variability. A number of alternatives have been proposed, including dynamic, magnetic, vacuum, electrostatic, and centrifugal seeding [51]. The leading option at this point seems to be vacuum seeding in a specially designed chamber, which is both more standardized and more effective in that it allows for rapid, operator-independent, and self-contained cell seeding [52].

Patient Selection

Although vascular tissue engineering holds the promise of many great advances over existing treatments for vascular disease, it remains a new field in only the infancy of its clinical application and so caution is warranted. Any clinical trial is by necessity a slow process and with pediatric patients that are so sick to begin with, research subject selection must proceed with care [53, 54].

Imaging Advances

Along with recent developments in vascular tissue engineering technology, many great strides have been made in the field of imaging in order to monitor TEVG development and function in vivo. These include the use of ultra-small particles of iron oxide to enable direct tracking of cells in vivo in order to study TEVG development in animal models using MRI and other imaging modalities [55, 56]. In the future these tissue engineering and imaging technologies will hopefully be translated to the clinic in parallel to aid in the evaluation of vascular grafts in human subjects.

Improving Clinical Outcomes

The findings of Shinoka's clinical trial in Japan are encouraging but also point to some of the possible issues and challenges with the use of vascular grafts in the pediatric population. Translational research groups can now return to animal models in the lab to improve TEVG outcomes [35]. Further investigation will identify critical mediators controlling the formation of stenosis in TEVG. An important goal is to use these discoveries to guide rational design of second-generation TEVG: first by targeting critical mediators of stenosis, the primary cause of TEVG failure, in order to design grafts with improved long-term patency; and second, by elucidating molecular mechanisms that control vascular neotissue formation in order to create cytokine or drug-eluting TEVG, which would not require cell seeding. The development of a TEVG that does not require cell seeding would improve the off-the-shelf availability of TEVG and dramatically increase its clinical utility.

Molecular Mechanisms of TEVG Stenosis

As previously described, Breuer and Shinoka have developed a method for fabricating TEVG scaffolds on a much smaller scale, enabling implantation in a murine model using microsurgical technique [28]. We have used the mouse model to investigate the cellular and molecular mechanisms underlying neovessel formation and the development of TEVG stenosis, the primary mode of graft failure in the Japanese clinical trial. In our mouse model, stenosis develops over a two-week time course [57]. Since seeded bone marrow mononuclear cells tend to prevent stenosis, we use an unseeded TEVG model as an approach to studying graft stenosis [57].

In our previous studies we demonstrated that the seeded cells are critical to the process of neovessel formation and function to inhibit the formation of TEVG stenosis. However we also noted that the seeded cells rapidly disappear from the TEVG suggesting that they exert their effect via a paracrine mechanism [31]. Additionally we showed that vascular neotissue arises from ingrowth of cells arising from the neighboring blood vessel, thus demonstrating that neovessel formation is a regenerative process [34]. Based on our discovery that neovessels arise from ingrowth of cells from the neighboring blood vessel wall and the finding that smooth muscle cells accumulate in occluded grafts, we hypothesized that endothelial-mesenchymal transition may play an important role in the process of neovessel formation and the development of TEVG stenosis.

It is well known that development of neointima underlies a number of common diseases including post-angioplasty and vascular graft restenosis, hypertension, and atherosclerosis [58, 59]. Despite decades of investigations, the origin of neointima still remains controversial with studies variously pointing to the role of medial smooth muscle

cell (SMC) proliferation [60], vessel wall inflammation [61] and adventitial angiogenesis [62]. Endothelial-mesenchymal transition (EMT) is a complex process by which ECs lose their attachments, differentiate and migrate. This phenomenon occurs normally during human development in organogenesis in the heart [63, 64]. EMT has recently been shown to participate in several pathological processes including cardiac fibrosis [65], fibrodysplasia ossificans [66] and portal hypertension [67]. Basement membrane damage in the kidney has been shown to cause epithelial cells to release cytokines that initiate EMT [68]. Fibrosis in a heart disease model was mediated by EMT and reduced by blocking the pathway [65]. EMT is thought to be driven by TGF-B in a Smad-dependent and independent manner [67, 69]. However, factors initiating EMT and suppressing its occurrence even in the normal vasculature remain poorly understood and are areas of active investigation [70].

Transforming growth factor B (TGF-B) is a multifunctional signaling molecule that regulates cell proliferation, differentiation, adhesion, migration, and apoptosis through signal transduction at two types of activin receptor-like kinases (ALKs) via Smad and MAPK pathways [71]. TGF-B exerts its effects by binding to and complexing type 1 and type 2 serine/threonine kinase transmembrane receptors. A ligand-induced heteromeric receptor complex results and the constitutively active type 2 receptor is then able to phosphorylate the type 1 receptor (TGFBR1). Once the type 1 receptor is activated, the Smad signaling molecules are recruited, form complexes, and translocate into the nucleus, where they are then able to regulate the transcription of specific gene targets [71]. Conflicting roles in angiogenesis and pathogenesis have been assigned to TGF-B as an inhibitor of proliferation and migration of ECs and enhancer of extracellular

matrix (ECM) accumulation and differentiation of mesenchymal cells into pericytes and smooth muscle cells [72]. A variety of studies have shown that TGF-B signaling plays essential roles in normal vessel development and physiology and that aberrant TGF-B signaling is key to a number of vascular disorders [64, 73].

Fibroblast growth factors (FGF) have also recently emerged as key molecular regulators of normal vascular biology [74]. Circulating and tissue-resident FGFs signal via cognate tyrosine kinase receptors that require an intracellular adaptor FRS2 for the initiation of most intracellular signals, including a critical MAPK pathway [75]. Experimental evidence using various in vitro models points to a role for FGFs in inhibition of TGF-B signaling [76]. Thus, FGF2 downregulates TGFBR1 expression and attenuates endothelial cell response to TGF-B [77] and antagonizes TGF-B-mediated smooth muscle actin expression [78]. In addition, FGF can revert TGF-B-induced EMT in epithelial cells via the MAPK pathway [79]. These observations suggest that loss of endothelial FGF signaling may lead to the upregulation of the TGF-B pathway and promotion of EMT.

Reports that EMT is driven by TGF-B suggest that blocking TGFBR1 signaling might inhibit EMT in our tissue engineered vascular graft and therefore improve TEVG patency [80]. We hypothesized that ECs in our TEVG undergo EMT driven by TGF-B to contribute to stenosis. To explore this process in our mouse model of TEVG formation, we used two endothelial cell lineage-tracing models to show that EMT occurs during neovessel formation. We also determined that TGF-B signaling is increased in occluded grafts. Based on this result we treated mice systemically with a small molecule TGFBR1 inhibitor (SB-431542) and showed improved patency. Finally, we used a novel system

for local delivery of this TGFBR1 inhibitor to prevent stenosis without cell seeding while maintaining normal neovessel formation.

Statement of Purpose

The development of an autologous tissue engineered vascular graft holds great promise for improving outcomes in congenital heart surgery. Currently used synthetic grafts are a significant source of morbidity and mortality, suggesting the need for alternative therapies. The first clinical trial evaluating the use of TEVG created by seeding autologous bone marrow-derived mononuclear cells onto biodegradable scaffolds showed that this approach is both safe and effective but that the primary mode of TEVG failure is graft stenosis. Preliminary data demonstrate that endothelial-mesenchymal transition driven by TGF-B, known to play a critical role in other vascular processes similar to TEVG vascular neotissue formation such as neointimal hyperplasia, may be involved in TEVG stenosis. The goal of this research project is to investigate the TGF-B-mediated mechanisms of vascular neotissue formation with a focus on mechanisms affecting formation of graft stenosis.

Hypotheses

1) TEVG stenosis results from TGF-B mediated endothelial-mesenchymal transition

2) Modulation of EMT can be used to reduce the incidence of stenosis in our TEVG

Specific Aims

1) To determine the relative contribution of EMT to TEVG stenosis

2) To modulate the pathways inhibiting or promoting EMT with the ultimate goal of reducing TEVG stenosis

Methods

(All methods performed by Daniel R. Duncan unless stated otherwise)

Scaffold Fabrication

TEVG scaffolds were constructed from a nonwoven polyglycolic acid (PGA) mesh (Concordia Fibers) and a co-polymer sealant solution of poly-L-lactide and –ε-caprolactone (P(CL/LA)) as previously described [28]. Briefly, tubular scaffolds were formed by compressing a 6.0 mm x 6.0 mm sheet of nonwoven P(CL/LA) felt between a 21-gauge stainless steel rod (to maintain the inner lumen) and a cylindrical cored-out polypropylene rod. The polymeric scaffolds were coated with a 50:50 copolymer sealant solution of poly–ε-caprolactone-l-lactide (263,800 Da; Absorbable Polymers International, Birmingham, Ala) dissolved at 5% wt/vol in dioxane. Scaffolds were snap frozen at –20°C for 30 minutes and then lyophilized overnight to remove solvent. SB-431542-eluting PGA-P(CL/LA) scaffolds were fabricated by substituting a 5% w/v P(CL/LA) containing 3 mg/ml SB-431542. Scaffolds were coated with fibrin and thrombin and sterilized at room temperature under UV light prior to implantation. Scaffolds used for *in vitro* drug release studies were not coated with fibrin or thrombin.

Bone Marrow Seeding of TEVGs

For scaffold seeding studies, bone marrow was collected from the femurs of syngeneic C57BL/6 mice. Following purification of the mononuclear cell component using Histopaque-1086 (Sigma) centrifugation, one million mononuclear cells were manually

pipetted onto the scaffold. The seeded scaffold was incubated in RPMI 1640 Medium (Gibco) overnight prior to implantation as previously described [28].

Generation of mice and embryos (Performed by Pei-Yu Chen, PhD)

Tie2-Cre (gift from W. C. Sessa, Yale University School of Medicine) transgenic mice or Cdh5-CreERT2 (gift from R. H. Adams, Cancer Research UK London Institute) transgenic mice were crossed with R26R-lacZ [B6,129-Gt(ROSA)26Sor^{tm1Sho}/J] (JAX SN:003309) or mT/mG [B6,129(Cg)-Gt(ROSA)26Sor$^{tm4(ACTB-tdTomato,-EGFP)Luo}$/J] (JAX SN:007676) mice to generate endothelial cell-specific reporter mice. For embryo analysis, timed matings were set up and the morning of the vaginal plug was considered as embryonic day 0.5 (E0.5). Embryos were genotyped by PCR analysis of the yolk sacs. PCR genotyping was performed by using the following primers: Rosa26 (5'-GCGAAGAGTTTGTCCTCAACC-3', 5'-AAAGTCGCTCTGAGTTGTTAT-3' and 5'-GGAGCGGGAGAAATGGATATG-3'), Tie2-Cre (5'-GCGGTCTGGCAGTAAAAACTATC-3', 5'-GTGAAACAGCATTGCTGTCACTT-3', 5'-CTAGGCCACAGAATTGAAAGATCT-3', and 5'-GTAGGTGGAAATTCTAGCATCATC C-3').

Tamoxifen administration (Performed by Pei-Yu Chen, PhD)

Tamoxifen was used to induce Cre expression in the Cdh5-CreERT2 mouse line. 100 mg tamoxifen (Sigma) was dissolved in 5 ml corn oil (20 mg/ml final concentration). The solution was mixed at 37°C overnight. Pups were pipette fed with 0.05 mg/g tamoxifen solution every other day for 8 times total.

TEVG Implantation (Surgical implantations performed primarily by Tai Yi, MD with assistance from Narutoshi Hibino, MD PhD and Yuji Naito, MD; pre-operative care, anesthesia, and post-operative care performed by Daniel R. Duncan)

All animal experiments were done in accordance with Yale institutional guidelines for the use and care of animals, and the institutional review board approved the experimental procedures described. TEVG scaffolds were inserted into the infrarenal inferior vena cava (IVC) of 8-10 week old, female mice as previously described [28]. Briefly, female C57BL/6 mice (6-8 weeks old, Jackson Laboratory, Bar Harbor, ME) were anesthetized with intraperitoneal injections of ketamine (100 mg/kg) (Hospira, Inc, Lake Forest, Ill) and xylazine (10 mg/kg) (Ben Venue Laboratories, Bedford, Ohio). After preparation and sterilization of the abdomen with betadine and alcohol, a midline laparotomy incision was made. The IVC was identified and exposed using an 18× dissecting microscope (Zeiss, Thornwood, NY), and the abdominal cavity bathed in heparinized solution (250 U/mL) (Baxter, Deerfield, Ill). Control of the IVC was obtained just inferior to the renal veins and superior to the iliac veins. TEVG scaffolds fabricated as above were implanted as infrarenal IVC interposition grafts via microsurgical technique. All anastomoses were preformed in an end-to-end technique using 10-0 monofilament nylon sutures (Sharpoint Lab Sutures, Calgary, Alberta, Canada) in interrupted stitches. Adequate hemostasis was achieved before closing the abdominal cavity. Graft recipients were recovered from surgery on warmed pads and evaluated for evidence of hind limb ischemia, paralysis, or acute graft thrombosis before being returned to their cages. All mice were maintained postoperatively without the use of any anticoagulation or antiplatelet therapy.

Tissue preparation and histology

Explanted grafts were pressure fixed in 10% formalin overnight and then embedded in paraffin or glycolmethacrylate using previously published methods [31]. Sections were stained with H&E or Gomori Trichrome by Yale Orthopedic Histology. For cryosection preparation, tissues were isolated from anesthetized mice, fixed 2 hr in 4% paraformaldehyde (PFA) at room temperature, cryoprotected in 30% sucrose overnight at 4°C and embedded in OCT (Tissue-Tek). Frozen tissue was then cut into 10-um-thick sections.

TEVG analysis

Graft luminal diameters were measured using Image J software. Stenosis was defined as greater than 50% decrease in luminal diameter. Critical stenosis was defined as 80% narrowing of the luminal diameter. Graft occlusion was defined as 100% narrowing of the luminal diameter. TGF-B positive cell area was measured using ImageJ software. Two separate sections of each explant were counterstained with hematoxalin and imaged at 400X magnification. The number of nuclei was then counted in five regions of each section and averaged. LacZ/SMA colocalized cells were quantified in the same manner using double immunofluorescent staining imaged under 60X confocal magnification using a Leica SP5 confocal microscope.

Whole mount X-gal staining

The expression of LacZ in scaffolds was detected by X-gal (β-glactosidase) staining using a beta-gal staining kit according to the manufacturer's instructions (MILLIPORE).

Following X-gal staining, the scaffolds were refixed, dehydrated, embedded in paraffin, and sectioned at 6 μm. The paraffin sections were then countered with eosin before being photographed.

Immunohistochemistry

Samples were fixed in 4% paraformaldehyde overnight, washed in 70% ethanol, embedded in paraffin and sectioned (6 um). Slides were dewaxed in xylene, antigen retrieval was performed by boiling for 20 min in citrate buffer, rehydrated and blocked in 5% normal goat serum in PBS-T for 30 min at room temperature. Primary antibodies included: β-galactosidase (Ab9361, Abcam), Calponin, clone hCP (C-2687, Sigma), CD31 (Ab28364-100, Abcam), smooth muscle α-actin (M0851, Dako), vWF (Dako), TGF-B (Ab53169, Abcam), VE-cadherin (C-19, Santa Cruz). Antibody binding was detected using appropriate biotinylated secondary antibodies, followed by binding of streptavidin-HRP and color development with 3,3-diaminobenzidine (Vector). Nuclei were then counterstained with hematoxylin. For immunofluorescence detection, a goat-anti-rabbit IgG-Alexa Fluor 568 (Invitrogen) or a goat-anti-mouse IgG-Alexa Fluor 488 (Invitrogen) was used with subsequent 4',6-diamidino-2-phenylindole nuclear counterstaining. Immunofluorescence was detected using a Leica SP5 confocal microscope. For each experiment, negative controls were used where sections were treated without primary antibody and stained with secondary antibodies only.

Quantitative Real Time PCR (qRT-PCR) (SYBR Green PCR performed by Pei-Yu Chen, PhD)

Explanted tissue grafts were frozen in OCT (Tissue-Tek) and each sectioned into forty 10um sections using Cryocut 1800 (Leica). Excess OCT was removed by centrifugation in water. RNA was then isolated using RNeasy plus Mini Kit (Qiagen) and converted to cDNA using iScript cDNA synthesis kit (Bio-Rad). Quantitative real-time PCR was performed using Bio-Rad CFX94 (Bio-Rad) by mixing equal amount of cDNAs, iQ SYBR Green Supermix (Bio-Rad) and gene specific primers. For TGFBR1, the following primer sequences were used: 5'-TCCCAACTACAGGACCTTTTTCA -3' and 5'-GCAGTGGTAAACCTGATCCAGA -3'. For Vimentin, the following primer sequences were used: 5'-CGGCTGCGAGAGAAATTGC-3' and 5'-CCACTTTCCGTTCAAGGTCAAG-3'. For mouse TGF-B we used the TaqMan detection system (Mm00441726_m1; Applied Biosystems) and followed the manufacturer's recommendations. All reactions were done in a 25 ul reaction volume in duplicate. Data were normalized to an endogenous control b-actin. Values are expressed as fold change in comparison to control.

sFGFR1-IIIc adenovirus administration (Tail vein injections performed with assistance from Nancy Kirkiles-Smith, PhD)

sFGFR1-IIIc adenovirus was administered as previously described [74] at a dose of $5x10^{10}$ viral particles per mouse 1 week prior to TEVG implantation by tail vein injection. Control mice were given equivalent volumes of sterile PBS. Serum level of sFGFR1-IIIc was measured by a Human IgG Subclass Profile kit (Invitrogen).

Mouse treatment with systemic SB431542

Mice treated systemically with TGFBR1 kinase inhibitor were treated with SB431542 hydrate (Sigma) in DMSO given by intraperitoneal injection twice a day from post-operative day 0 to post-operative day 14 at a dose of 10 mg/kg. Control mice were treated with equivalent volumes of sterile DMSO.

Microparticle synthesis and characterization (Performed by Joseph Patterson, YSM II with guidance from Tarek Fahmy, PhD)

SB-431542 (Sigma-Aldrich Cat. No. S4317) was encapsulated in avidin-coated PLGA microparticles using a modified oil/water single emulsion technique [81]. Briefly, 5 mg of drug and 100 mg PGLA (50/50 monomer ratio, Durect Corp. Cat. No. B0610-2) dissolved in 2 ml chloroform and 200 ul DMSO were added dropwise with vortexing to 4 mL of aqueous surfactant solution containing 2.5 mg/mL polyvinyl alcohol (PVA) (Sigma-Aldrich Cat. No. 363138) and 2.5 mg/mL avidin–palmitate bioconjugate to create an emulsion containing microsized droplets of polymer/solvent, encapsulated SB-431542 and surfactant. Solvent was removed by magnetic stirring at 20C; hardened microparticles were then washed 3× in DI water and lyophilized for long-term storage at -20C. Control avidin-coated PLGA microparticles were synthesized as above without SB-431542. Microparticle size and morphology were analyzed via scanning electron microscopy (SEM). Samples were sputter-coated with gold under vacuum in an argon atmosphere using a sputter current of 40 mA (Dynavac Mini Coater, Dynavac, USA). SEM analysis was carried out with a Philips XL30 SEM using a LaB electron gun with an accelerating voltage of 10 kV.

Preparation of adhesive peptide tether (Performed by Joseph Patterson, YSM II with guidance from Tarek Fahmy, PhD)

Poly-L-lysine-LC-LC-biotin (pLLB) was synthesized and used as an adhesive peptide tether to enhance loading of PGA-P(CL/LA) scaffolds with avidin-coated microparticles. 1.66 mg EZ-Link sulfo NHS-LC-LC-biotin was reacted with 10 ml of a 0.1 mg/ml solution of poly-L-lysine (MW 70,000-150,000, Sigma-Aldritch Cat. No. P4707) in 1x PBS for 2 hours at 4C, dialyzed in 1x PBS for 72 hours, and stored at 4C.

Loading of TEVG scaffolds with SB-431542-eluting microparticles (Performed by Joseph Patterson, YSM II with guidance from Tarek Fahmy, PhD)

Nonspecific adsorption of avidin-coated PLGA microparticles to PGA-P(CL/LA) scaffolds not treated with pLLB was titrated by incubating scaffolds trimmed to 5 mm in axial length with 1 ml of 1, 5, or 10 mg/ml of microparticles in 1x PBS for 10, 30 or 60 minutes. Particle-loaded TEVG scaffolds were immediately snap frozen in liquid nitrogen and lyophilized for 6 hours before imaging. Scaffold loading efficiency was determined with ImageJ software (Image Processing and Analysis in Java, National Institute of Health, Bethesda, MD) from three SEM images per scaffold cross section, inner surface, and outer surface by calculating the mean surface density of particles. The effect of scaffold pretreatment with pLLB on scaffold loading efficiency was assessed from particle loading density as above after PGA-P(CL/LA) scaffolds were incubated with 1 ml of 0.01, 0.1 or 1 mg/ml pLLB for 60 minutes on a rotary shaker, washed 3 times with dH2O, incubated with 1 ml 5 mg/ml avidin-coated PLGA microparticles on a rotary shaker, washed 3 times with dH2O, snap frozen in liquid nitrogen, lyophilized for

6 hours, and imaged by SEM. For in vitro and in vivo studies, PGA-P(CL/LA) scaffolds were incubated with pLLB for 30 minutes at 20C on a rotary shaker, washed 3 times with dH2O, incubated with 5 mg/ml empty or SB-431542-eluting avidin-coated PLGA microparticles for 30 minutes on a rotary shaker, washed 3 times with dH2O, snap frozen in liquid nitrogen, and lyophilized for 6 hours before storage in a dessicator.

Characterization of SB-431542 release from microparticles and scaffolds (Performed by Joseph Patterson, YSM II)

Total encapsulation was approximated as the amount of SB-431542 released over a 14-day period. Percent encapsulation efficiency was calculated as total encapsulation divided by maximum theoretical encapsulation. 5 mg of avidin-coated PLGA microparticles containing SB-431542, PGA-P(CL/LA) scaffolds trimmed to 5 mm axial length and treated with pLLB and SB-431542-eluting microparticles as above, and SB-431542-eluting PGA-P(CL/LA) scaffolds trimmed to 5 mm axial length were incubated with 400 µl 1x PBS in 2 ml microcentrifuge tubes in triplicate on a rotary shaker at 37C. Samples were removed at time points of 1, 2, 4, 8, 12, 24, 36, 48, 72, 96, 120, 168, 240, and 336 hours and centrifuged at 13200 RPM for 10 ten minutes. 300 µl of supernatant was drawn and replaced with 300 µl 1x PBS. Concentration of SB-431542 in supernatant diluted with 600 µl 1x PB was determined by spectrophotometry at 320 nm in a quartz cuvette.

Bioactivity of encapsulated SB-431542by p-SMAD immunoblot (Performed by Muriel Cleary, MD)

SB-431542 was released into 1 ml 1x PBS from 10 mg avidin-coated PLGA microparticles and one untrimmed SB-431542-eluting PGA-P(CL/LA) scaffold in 2 ml microcentrifuge tubes on a rotary shaker at 37C. At 48 hours, samples were centrifuged at 13200 RPM for 10 ten minutes, supernatants were collected and analyzed by spectrophotometry at 320 nm. SB-431542 concentrations were adjusted to 10 μM by dilution with 1x PBS. 3T3 human fibroblasts were plated at 500,000/well on a 6-well plate and stimulated at confluence with 700 ul 10 μM SB-431542 in PBS eluted from particles or scaffolds, a stock solution of 10 or 1 μM SB-431542 containing <1% DMSO, 1 or 1x PBS. After 30 minutes at 37C, cells were washed with warm PBS and stimulated with 200 ul 2 ng/ml recombinant human TGF-β1 (BD Biosciences, Cat. No. 354039) for 1 hour at 37C. Cells were lysed with ice cold RIPA lysis buffer containing phosphatase and proteinase inhibitors (PhosSTOP and cOmplete mini, Roche Applied Science, Cat. No. 04906845001 and 04693116001). Cell lysates were collected in 200 μl ice cold 1x PBS by scraping, vortexed for 15 seconds, agitated on a rotary shaker at 4C for 30 minutes, and centrifuged at 12000 RPM for 15 minutes at 4C. Supernatant protein concentrations were determined by DC protein assay (Bio-Rad Life Science, Hercules, CA) and protein samples were separated by gel electrophoresis with a 12% polyacrylamide gel. Samples were transferred to a PVDF membrane, blocked with 5% milk, and probed with primary rabbit monoclonal antibody against phosphorylated SMAD-2 (ser426/ser428, Cell Signaling Technology, Cat . No. 3010) and secondary goat anti-rabbit IgG (Cell Signaling Technology, Cat. No. 7074). The gel was stripped in stripping buffer (50 ml 62.5 mM Tris-HCl, 2% SDS, 100 mM β-mercaptoethanol) for 40 minutes at 50C with agitation and reprobed with anti-SMAD2/3 as a loading control. The

proteins were visualized with ECL exposure on HyBlot x-ray film and analyzed for band density.

Statistical analysis

Data are the mean ± standard deviation. Statistical comparisons between groups were performed by the one-way analysis of variance followed by the Student's t-test. Patency rates were compared using the Fisher's Exact Test. *P* values less than 0.05 were considered significant.

Results

EMT occurs during neovessel formation

We performed endothelial cell lineage-tracing studies in order to map the fate of the endothelial cells in our TEVG as they transitioned from endothelial cells to mesenchymal cells while maintaining expression of LacZ. Implantation of our graft into a Tie2-Cre transgenic mouse model (n=9) demonstrated co-localization of LacZ with SMA and Calponin, mesenchymal cell markers, suggesting the occurrence of EMT (Figure 4a). We validated these studies using a Cdh5-CreERT2 transgenic mouse model (n=7) and again demonstrated co-localization of LacZ with SMA and Calponin, confirming our previous results (Figure 4c). In both models, LacZ-positive cells are found throughout the entire neointima and have lost expression of the endothelial cell marker CD31.

EMT contributes to occlusion in TEVG

We quantified the degree of EMT in our TEVG by performing confocal analysis and cellular quantification of TEVG samples double stained for LacZ and SMA. In a comparison of patent to occluded grafts we found co-localization of both markers in 38-51% of smooth muscle cells (Figure 4b, d) suggesting that EMT significantly contributes to occlusion in our TEVG.

Figure 4: Cell fate mapping demonstrating EMT using two transgenic models

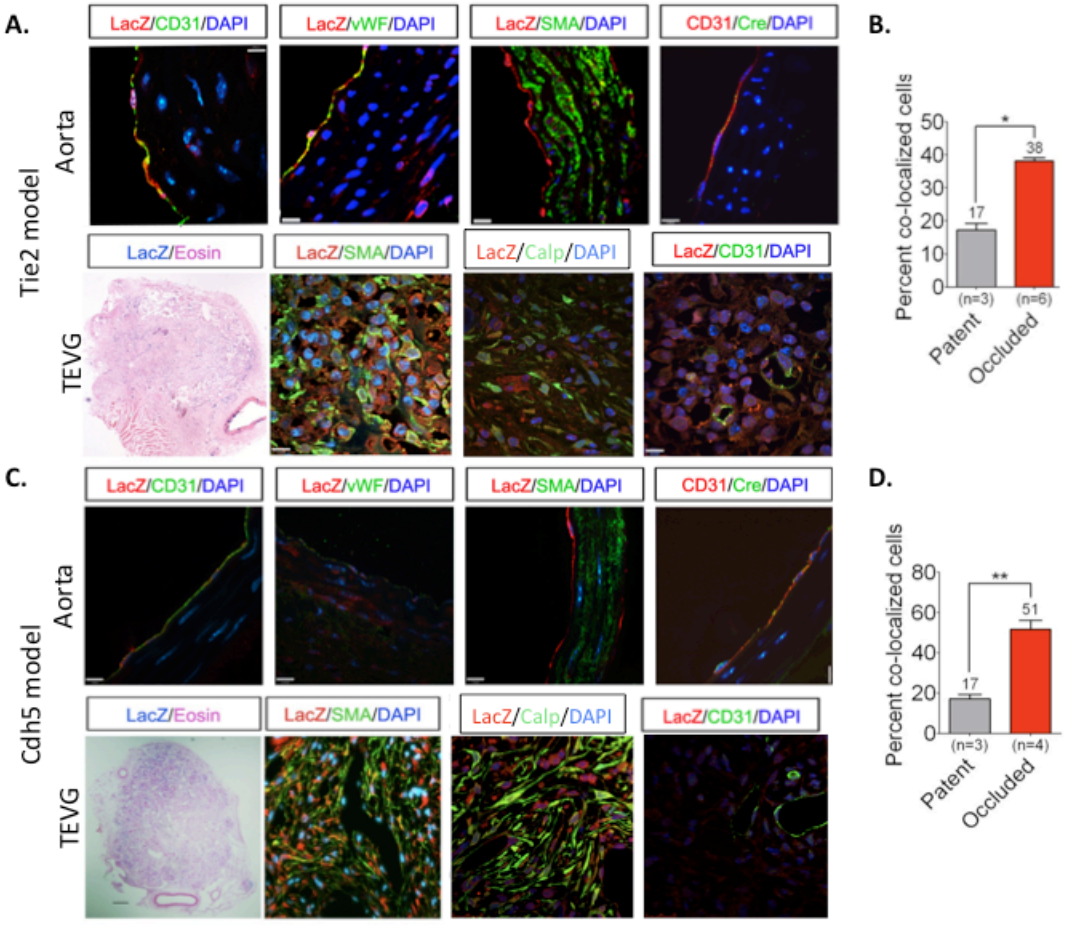

Figure 4: Cell fate mapping demonstrating EMT using two transgenic models. A. Tie2 model with first row images of model development showing specificity of lineage tracing using LacZ in aorta and second row showing lineage tracing images in occluded TEVG in Tie2 mice. (Scale bar: 12 μm). B. Quantification demonstrating that EMT significantly contributes to TEVG stenosis in the Tie2 model. (*$p<0.05$). C. Cdh5 model with first row images of model development showing specificity of lineage tracing using LacZ in aorta and second row showing lineage tracing images in occluded TEVG in Cdh5 mice. D. Quantification demonstrating that EMT significantly contributes to TEVG stenosis in the Cdh5 model, replicating the results shown with the Tie2 model. (**$p<0.01$).

Expression of TGF-B and TGFBR1 are associated with EMT

At 2 weeks, patent TEVGs have an organized neovessel structure with an intima consisting of vWF positive endothelial cells and a media consisting of SMA positive

smooth muscle cells. In contrast, occluded grafts show SMA positive smooth muscle cell accumulation (Figure 5a). Both immunohistochemical staining and qRT-PCR demonstrate increased expression of TGF-B in unseeded grafts when compared to grafts seeded with bone marrow mononuclear cells, a procedure previously shown to dramatically improve graft patency (Figure 5b, c). In an analysis of our unseeded grafts, qRT-PCR showed increased TGF-B, TGFBR1, and vimentin expression in occluded grafts at 2 weeks when compared to 1 week samples, the time course over which stenosis has been shown to take place in our model (Figure 5d).

Figure 5: TGF-B signaling plays a role in TEVG stenosis

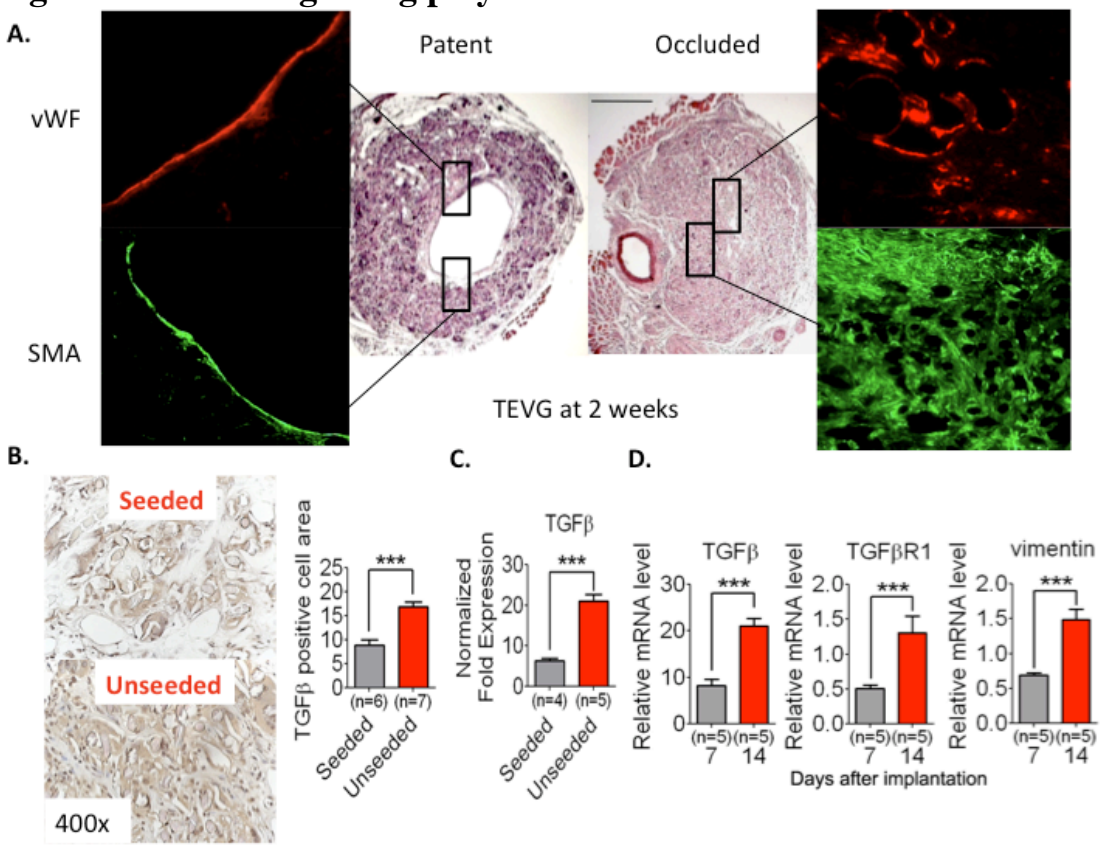

Figure 5: TGF-B signaling plays a role in TEVG stenosis. A. Smooth muscle cells are implicated in stenosis. B. Higher TGF-B expression in occluded grafts by IHC. C. Higher TGF-B expression in unseeded grafts compared to seeded grafts by qRT-PCR. D. Higher expression of TGF-B and TGFBR1 at 2 weeks in unseeded TEVG by qRT-PCR. (***p<0.001).

Regulation of TGFBR1 by FGF Signaling in TEVGs

To determine if transcriptional regulation of TGFBR1 by FGF signaling plays a role in TEVG stenosis, we systemically expressed a soluble FGF trap (Ad-sFGFR1-IIIc) that has been previously demonstrated to virtually shutdown FGF signaling [74] one week before implantation of TEVG seeded with bone marrow mononuclear cells, a procedure known to dramatically improve the graft patency (Figure 6a, upper panel). Two weeks after graft implantation, there were significantly higher TEVG neointima burden and stenosis rate in mice injected with Ad-sFGFR1-IIIc (n=15) compared to saline-injected control mice (n=15) (Figure 6b, c). Serum was analyzed for sFGFR1-IIIc by sandwich ELISA to confirm FGF trap expression in treated mice (Figure 6a, lower panel).

Figure 6: FGF blockade using a viral trap to upregulate TGF-B signaling increases stenosis

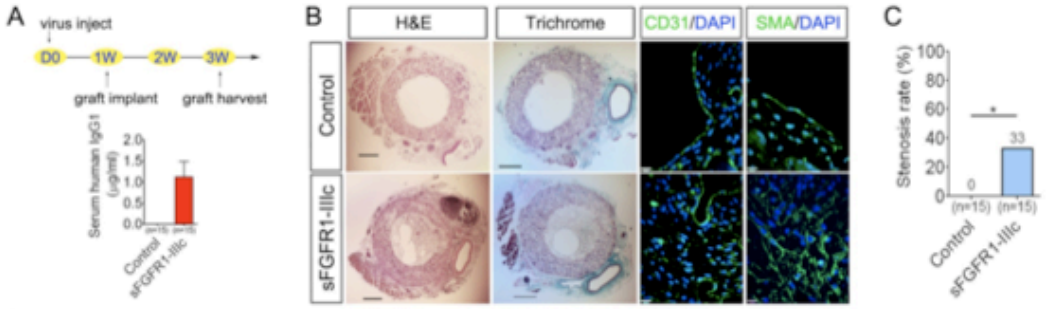

Figure 6: FGF blockade using a viral trap to upregulate TGF-B signaling increases stenosis. A. Schematic representation of the seeded TEVG implantation schedule for mice with tail vein injection of sFGFR1-IIIc virus or saline. Serum was analyzed for sFGFR1-IIIc by sandwich ELISA. Data shown represents mean ± SD from 2 wells per group. B. Qualitative IHC demonstrating patent control and stenosed virus-treated neovessel histology. C. TEVG stenosis rate in control and sFGFR1-IIIc treated mice. (*$p<0.05$ compared to control).

Inhibition of TGFBR1 inhibits stenosis but does not block neovessel formation

We treated mice with the TGFBR1 inhibitor SB-431542 at 10 mg/kg twice daily by intraperitoneal administration for 2 weeks following implantation of our TEVG (n=16). Matched control mice received intraperitoneal injection of sterile DMSO (n=25). Results of these studies showed that TGFBR1 inhibitor treatment increases TEVG luminal diameter and graft patency at 2 weeks in unseeded grafts (Figure 7a). Drug treatment also allows for proper neotissue creation with an organized CD31-positive endothelial cell layer lining an SMA-positive smooth-muscle cell layer in contrast to the typical untreated control graft that occludes as a result of accumulation of SMA-positive smooth muscle cells (Figure 7b).

Figure 7: Systemic SB431542 treatment reduces TEVG stenosis by blocking EMT

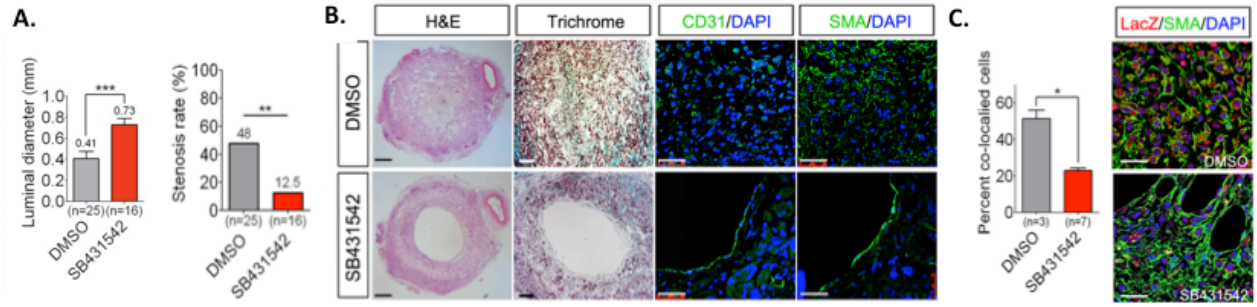

Figure 7: Systemic SB431542 treatment reduces TEVG stenosis by blocking EMT. A. SB431542 experiment with quantitative analysis of patency and luminal diameter. B. Qualitative IHC demonstrating normal neovessel histology. C. Results of experiment combining Tie2 lineage tracing model with SB treatment to demonstrate that drug treatment significantly reduces the occurrence of EMT in occluded TEVG. (*p<0.05; **p<0.01; ***p<0.001).

TGFBR1 inhibition blocks EMT

In order to unravel the mechanism by which SB431542 treatment prevents TEVG stenosis, we treated mice from the Tie2 lineage-tracing model (n=10) with TGFBR1

inhibitor drug and then performed confocal analysis and cellular quantification of TEVG samples double stained for LacZ and SMA. Results of these studies showed that drug treatment improves patency by significantly reducing the occurrence of EMT as demonstrated by a significant reduction in LacZ-positive smooth muscle cells in occluded grafts in drug treated mice (Figure 7c).

Local delivery of TGFBR1 inhibitor inhibits stenosis without cell seeding and maintains normal neovessel formation

We developed a novel microparticle system for local delivery of the TGFB R1 inhibitor SB431542 in order to minimize possible effects of systemic delivery (Figure 8a). We characterized this system to show that there is steady release of the drug across the full 2-week time course during which the grafts are implanted (Figure 8b), and showed that the released drug maintains its biologic activity (Figure 8c). We also developed a simpler local drug delivery system by which the TGFBR1 inhibitor was added to the solvent used to make the grafts and again demonstrated a favorable release profile and continued biologic activity of the released drug (Figure 8b, c). We then implanted both types of drug-eluting grafts in our mouse model (n=10 for drug in microparticles, n=24 for drug in solvent) and compared their patency to control grafts with empty microparticles (n=10) or control grafts (n=25). Results of these studies showed that local drug delivery significantly increases patency at 2 weeks in unseeded grafts and also enables neotissue creation (Figure 8d, e).

Figure 8: Local delivery of SB431542 reduces TEVG stenosis

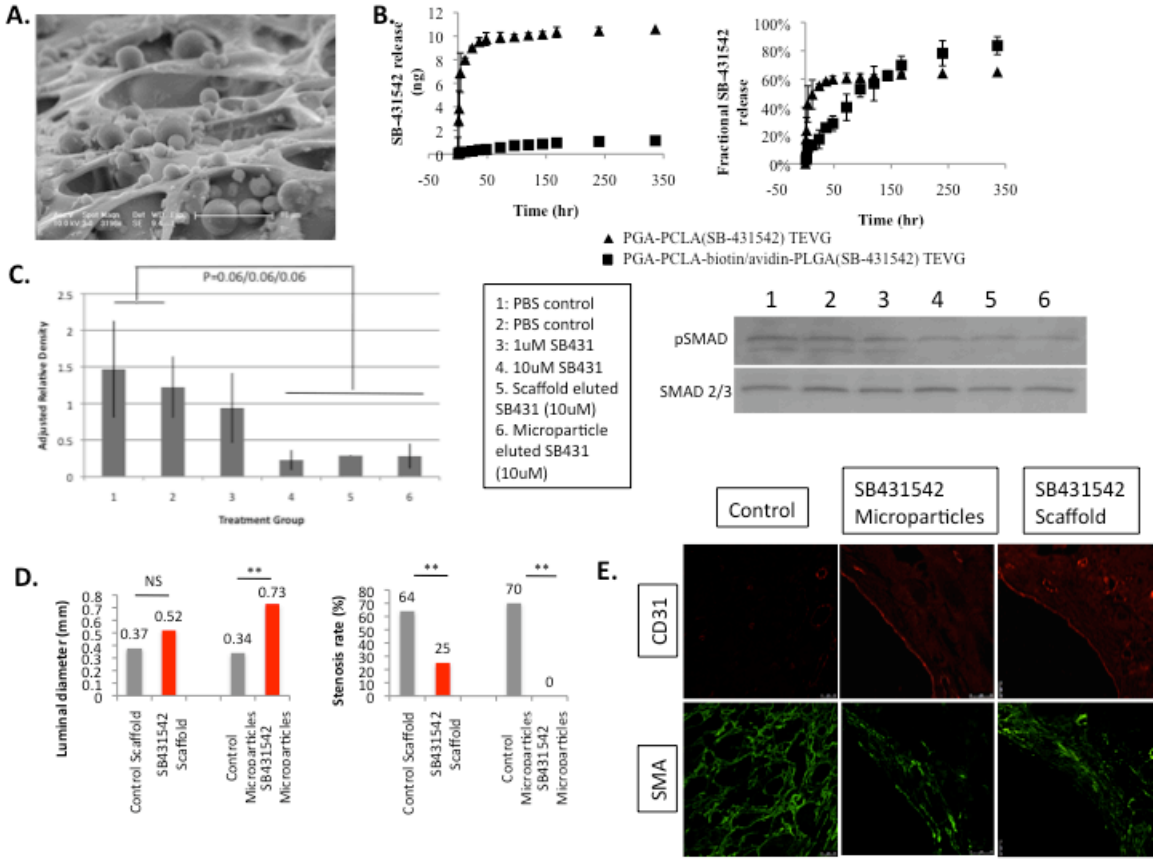

Figure 8: Local delivery of SB431542 reduces TEVG stenosis. A. SEM of particles seeded on inner lumen of TEVG. B. Absolute and fractional release of SB-431542 from (▪) tethered PLGA microparticles and (Δ) PCLA phase of a PGA-PCLA TEVG scaffold. C. Western for Smad2 and pSmad2 demonstrating bioactivity of SB431542 released from both types of scaffold. E. Quantitative analysis of patency and luminal diameter. (**p<0.01; NS = not significant) F. Qualitative IHC demonstrating normal neovessel histology.

Discussion

We have adopted a bench to the bedside and back approach to developing vascular tissue engineering technology. Our previous clinical studies have demonstrated the feasibility of using this technology in humans while also highlighting its unique advantages and in particular the growth capacity of our tissue engineered vascular graft [25]. These studies have also informed us that the primary graft related complication in humans is stenosis and thus have served to focus our ongoing laboratory investigations [22]. The development and use of the mouse IVC interposition model that recapitulates neovessel formation in a manner similar to that observed in large animal studies and human studies has provided us with a powerful tool for investigating neovessel formation [28, 31]. These laboratory studies provide us with clinically relevant information that we can apply to the rational design of improved second generation TEVGs [82]. This goal is predicated on improving our understanding of the cellular and molecular mechanisms underlying neovessel formation and the development of TEVG stenosis.

The results of the present study suggest that endothelial-mesenchymal transition under the control of TGF-B is a significant mediator of stenosis in our tissue engineered graft model and that modulation of this pathway might be useful in the development of improved tissue engineered vascular grafts for clinical use. The current study is the first to show endothelial-mesenchymal transition plays a role in TEVG stenosis. This was determined using two distinct and robust endothelial cell lineage-tracing models [66, 83]. For the first model, Tie2-Cre is constitutively active during embryonic development while in the second model the Cdh5-Cre construct was induced by tamoxifen on the second postnatal day. In both cases approximately half of neointimal smooth muscle cells

inside the TEVG were marked with LacZ, suggesting endothelial cell origin. Therefore, both models showed that stenosed and occluded grafts have a significantly higher percentage of cells that have transitioned from endothelial cells to smooth muscle cells as shown by loss of endothelial markers and gain of smooth muscle markers.

Endothelial-mesenchymal transition is a complex process that has been shown to have important roles in normal development and in other models of disease, including tumor metastasis and cardiac and renal fibrosis [63, 84]. EMT results from a complicated signaling process that is now better understood to be driven by TGF-B [84]. TGF-B has been discussed as playing an important role in restenosis in variety of other vascular models [85]. We confirmed that TGF-B is involved in stenosis in our TEVG mouse model by using both immunohistochemical analysis and qRT-PCR to show increased TGF-B and TGFBR1 expression at 2 weeks compared to 1 week in unseeded grafts and in a comparison between seeded and unseeded grafts. Both of these are clinically significant results since 2 weeks has been shown to be the key time point for the development of stenosis in our mouse model and graft seeding is a procedure known to increase patency in our graft [57].

Based on the finding that EMT and TGF-B play a role in graft stenosis along with the understanding that FGF signaling inhibits TGF-B signaling, we used a soluble FGF-trap adenovirus to block FGF signaling in our mice [74]. In this way we were able to at least indirectly increase TGF-B signaling to determine the effects of this change on graft patency. As expected, this resulted in increased stenosis in our TEVG, providing more evidence that TGF-B signaling is intimately involved in graft stenosis.

Next, based on the results of our lineage-tracing models along with the well-known link between EMT and TGF-B signaling, we were able to rationally select a small molecule inhibitor of TGFBR1 and test its effect on stenosis in our TEVG. SB-431542 is a selective small molecule inhibitor of ALK5 that antagonizes TGF-B receptor type 1 and has been shown to block TGF-B mediated fibrosis [86-88]. We started with a systemic twice-daily treatment approach for mice that were implanted with our grafts. Results of these experiments revealed that the TGFBR1 inhibitor increased patency in our graft while allowing for normal neotissue formation with organized smooth muscle and endothelial cell layers. Combining this drug treatment with the power of our EC lineage-tracing model, we were importantly able to show that treatment with this drug specifically inhibits EMT in our graft. Therefore, we can conclude that inhibition of TGFBR1 signaling in our TEVG decreases stenosis specifically by decreasing the occurrence of EMT.

In order to further refine our approach to drug-based modulation of EMT and reduce the possibility of systemic side effects, we developed two local delivery methods for the TGFBR1 inhibitor. The first involved dissolving the drug in dioxane, the solvent used in the standard preparation of our TEVG, and thus incorporating it into the graft as the PCLA polymer is applied to the PGA fibers in graft production. In this case, the drug was released off the graft as the TEVG itself degraded by hydrolysis. The second approach involved developing a novel microparticle system in which the same drug was encapsulated in PLGA microparticles and bound in place by an avidin-biotin molecular tether. In this setting, the drug was eluted into the circulation as the microparticles degraded over time. Both approaches enabled local delivery of the TGFBR1 inhibitor at

greatly lowered doses and when grafts made with these drug delivery systems were implanted in mice we again saw a significant improvement in stenosis at two weeks along with normal neotissue formation. Interestingly, the drug worked to prevent stenosis with each of these delivery methods even though both the rate of release and total amount released differed between the two approaches. In using both a simple approach and a more sophisticated method, we have importantly developed two different platforms that could be adapted for the use of a variety of other drugs that work at varying time points to prevent stenosis.

TGF-B is a complex signaling molecule that has been shown to play a variety of roles throughout development, physiology, and disease. Other groups have shown that a possible effect of TGF-B is to inhibit MCP-1 expression [89, 90] and that macrophages also have other mechanisms for counteracting the effects of TGF-B [91]. Previous studies in our lab have shown the importance of the cytokine MCP-1 in coordinating the process of neotissue formation, likely by attracting the appropriate monocyte populations to the graft that direct or participate in tissue regeneration [31]. An interesting formulation of the results of this study is that perhaps by blocking the effects of TGF-B in our grafts we are allowing MCP-1 and the monocytes and macrophages it attracts to properly coordinate neotissue formation.

In our previous studies we have shown in the formation of TEVG that the degree of macrophage infiltration into the TEVG is correlated with the degree of development of TEVG stenosis is an immune mediated phenomena [57]. We previously evaluated macrophage depletion using clodronate liposomes as a potential strategy for inhibiting the development of TEVG stenosis. Using this strategy we were able to inhibit stenosis;

however, we also noted inhibition of neotissue formation, including absence of endothelial cell and smooth muscle cell formation on the luminal surface of the scaffold in addition to a paucity of ECM production. Based on these studies we concluded that macrophage infiltration into the TEVG scaffold was essential for vascular neotissue formation [57]. In contradistinction, inhibition of EMT by blocking TGFBR1 inhibited the formation of TEVG stenosis without blocking vascular neotissue formation and neovessel formation, a significant accomplishment in our path to developing a better tissue engineered vascular graft.

While administration of SB431542 was well tolerated in our study as demonstrated by no alteration in the surgical morbidity and mortality rates and maintenance of normal growth of our animals during the course of our study, potential systemic side effects are clearly of concern [92]. In order to minimize the systemic toxicity of TGFBR1 inhibition we investigated the use of local controlled release as a strategy for minimizing systemic toxicity. Our data suggests this is a viable strategy, resulting in a decreased incidence of stenosis and excellent neovessel formation as evidenced by normal neovessel histology. We used both a straightforward and a more sophisticated approach and in so doing also developed two novel platform technologies for the local delivery of drug in our TEVG. These would enable the use of a variety of small molecules to guide and improve neovessel development in vivo.

Limitations of the Present Study

There are several limitations of the present study, which must be taken into account when considering the results. While our study has only been performed in a

tissue-engineered model, it likely has applications to neointima formation and stenosis in other models. Intravascular stenosis following TEVG implantation is typical of other vascular injury restenosis models [93] but this needs to be verified in other settings such as intraluminal balloon injury or carotid artery ligation. Similarly, the current study only included experiments with our mouse model of a tissue engineered vascular graft. Further work with large animal models as our lab has pursued in the past would need to be performed to get a better sense of whether these results can be applied to the clinical trial in humans [32]. With regards to our transgenic mouse model experiments, we nicely showed that drug treatment reduces EMT using the Tie2 endothelial cell lineage-tracing model but this will also need to be verified using our Cdh5 lineage-tracing model. Similarly, local drug delivery will need to be used in the lineage-tracing setting to show that it has the same effect to block EMT. Additionally, in our use of the FGF trap virus we unfortunately present only indirect evidence that increased TGF-B signaling leads to increased stenosis in our TEVG. An important follow-up experiment will require some mode of treatment with TGF-B to show that this results in an increase in stenosis, whether by viral expression of TGF-B or systemic treatment with exogenous TGF-B. Additionally, we primarily focused on the endothelial cell markers CD31 and vWF and the smooth muscle cell markers SMA and Calponin in analyzing the occurrence of EMT in our TEVG model. Further investigation would involve confirming these results with EMT quantification using additional cell phenotype markers. Finally, in regard to both methods of local drug delivery, the release profiles were performed in vitro, and so it must be taken into account that the results are not necessarily representative of what occurs in vivo once the grafts are implanted in the mice.

Next Steps

Although the current study enables significant advances in our understanding of mechanisms of TEVG stenosis, many unanswered questions remain to be explored to further clarify the exact role of EMT in this process and how best to modulate this pathway to create a better TEVG for clinical use.

It has been shown that the EMT pathway diverges down two different signaling modalities based on whether it involves signaling through the Smads [84, 85]. Our approach has essentially focused on the Smad-dependent arm of this pathway by using the TGFBR1 inhibitor SB-431542, but an important next step would be to further unravel the EMT pathway as it occurs in our TEVG model and determine the Smad-dependence of the EMT pathway in TEVG stenosis.

An additional area of future investigation would involve determining where the endothelial cells that participate in EMT in our graft originate. The traditional view of this is that the endothelial cells migrate from the vessel wall, from cell populations in either the intima or the adventitia. However, more recent studies suggest the possibility of a circulating endothelial cell progenitor origin for these cells [94]. More sophisticated cell tracking experiments will be necessary to unravel these origins.

Most of our analysis has been focused at two weeks post-implantation since we have previously shown that this is the key time point for establishment of TEVG patency or stenosis [57]. However, we need to more deeply understand the exact timing of stenosis and establish a finer timeline for the occurrence of EMT in TEVGs to know if treatment is necessary and exactly when it would be most beneficial. This would involve looking at earlier time-points to determine the role of EMT in the acute phase of

neotissue formation and following mice for longer than 2 weeks with and without drug treatment to make sure the improvement in patency can be sustained.

Intriguingly, there are a variety of other possible inhibitors of the TGF-B signaling pathway including the ACE-inhibitor Losartan and several other drugs already in clinical use [95-99]. A better understanding of which components of the EMT pathway are involved in the formation of stenosis will enable us to continue to take a rational approach to selecting and screening drugs for their efficacy in preventing graft stenosis. Exploring other possible small molecule inhibitors of EMT might help us to find more safe and effective drugs for use in our TEVG.

An additional important area of investigation will be to use transgenic mouse models to better define the role of TGFBR1 and other components of the TGF-B signal transduction pathway in TEVG stenosis. In the long term we hope to use an endothelial cell-specific TGFBR1 conditional knockout mouse [64] to show that defective TGFBR1 signaling leads to decreased graft stenosis by means of a reduction in EMT. Such a result would provide more robust support for our current findings and enable a variety of other experiments to further unravel the mechanisms of stenosis in our TEVG.

Conclusions

The importance of EMT to neointima formation is only now beginning to be appreciated. Early studies have demonstrated the presence of mesenchymal-type cells in human restenotic lesions [93] but the significance and the origin of these cells have not been well defined. The current study ties together several lines of evidence including the importance of endothelial cell proliferation in neointima formation [62], the role of TGF-

B signaling in EMT [62], and the ability of FGF to affect TGF-B-induced EMT [79]. The inhibition of neointima formation by interference with this signaling pathway may open new therapeutic venues in a variety of clinical settings.

In this study we demonstrated that the process of endothelial-mesenchymal transition is involved in the formation of neotissue stenosis and occlusion in tissue engineered vascular grafts. Furthermore, we demonstrated that modulation of EMT by means of blockade of the TGF-B signaling pathway is a viable strategy for inhibiting the formation of TEVG stenosis and that this can also be done equally well by local drug delivery. There is certainly a complex route to stenosis in TEVGs and EMT is not necessarily the only or even the dominant pathway in this process but the results of this study suggest that EMT is at least one component of this phenomenon in our TEVG model.

Perhaps the most interesting finding of our study is that local delivery of TGFBR1 inhibitor could be used in place of cell seeding. The ability to perform cell-free tissue engineering has significant implications which would dramatically improve the clinical utility of this technology by enabling off the shelf availability without the need for cell harvesting, cell isolation, cell seeding, or incubation in vitro, all of which increase the potential complications associated with the use of this technology in humans. In addition, the notion that we can create a man-made device that induces organized tissue regeneration holds great promise for other regenerative medicine applications.

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