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Paul S. Bagi
Yale School of Medicine, paul.bagi@yale.edu

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ISOLATION OF BONE MARROW MONONUCLEAR CELLS FOR FABRICATION OF TISSUE-ENGINEERED VASCULAR GRAFTS: EVALUATION OF TWO METHODS

A Thesis Submitted to the
Yale University School of Medicine
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

by

Paul S. Bagi

2015

ISOLATION OF BONE MARROW MONONUCLEAR CELLS FOR FABRICATION OF TISSUE-ENGINEERED VASCULAR GRAFTS: EVALUATION OF TWO METHODS. Paul S. Bagi, Hirotsugu Kurobe, Shuhei Tara and Christopher K. Breuer. Division of Pediatric Surgery, Department of Surgery, Nationwide Children's Hospital, Columbus, OH. (Sponsored by Edward L. Snyder, Department of Laboratory Medicine, Yale University, School of Medicine, New Haven, CT).

Tissue engineered vascular grafts (TEVGs) are useful in the surgical treatment of congenital heart defects. The development of TEVGs requires seeding of scaffolds composed of biodegradable polymers with bone marrow-derived mononuclear cells (BM-MNCs). The most common method used to isolate BM-MNCs involves density centrifugation in Ficoll. This process requires an International Organization for Standardization (ISO) class 7 clean room, is labor intensive, time intensive, and susceptible to operator variability. A recently developed filtration-based method for BM-MNC isolation uses a closed, sterile, and disposable system that removes the need for a clean room, decreases processing time, and is operator-independent. This study compared the efficacy of each method of BM-MNC isolation by evaluating the viability of cells recovered using each method, and by assessing the biologic and structure equivalence between neo-vessels created from scaffolds seeded using cells isolated by either method.

BM-MNCs were isolated from the bone marrow of immunocompetent syngeneic C57BL/6 wild type mice by either density centrifugation in Ficoll or using a filter-based

method. The cells were seeded onto scaffolds fabricated from a polyglycolic acid (PGA) mesh coated with a 50:50 copolymer sealant of poly-L-lactide-co-ε-caprolactone. Seeded scaffolds were incubated overnight and then implanted as inferior vena cava (IVC) interposition grafts in 10-week-old wild type mice (n = 23 for each group). Grafts were explanted at 2 weeks post-implantation for analysis.

Significantly greater total (filter: 44.3 ± 12.6x10^6 cells/mouse versus density centrifugation: 24.8 ± 8.8x10^6 cells/mouse, p=0.02) and viable (filter: 32.8 ± 6.7x106 cells/mouse versus density centrifugation: 20.6 ± 8.7x106 cells/mouse, p=0.04) BM-MNCs were isolated using filtration versus density centrifugation-based isolation. There was no significant difference in graft patency (filter: 78% patency versus density centrifugation: 87% patency, p=0.7), luminal diameter (filter: 633 ± 131 μm versus density centrifugation: 620 ± 82.9 μm, p=0.72) or neointimal thickness (filter: 37.9 ± 11.2 μm versus density centrifugation: 37.9 ± 7.8 μm, p=0.99) between groups at explantation. There was also no significant difference in quantitative macrophage infiltration between the two methods at explantation (filter: 1887 ± 907.7 cells/mm^2 versus density centrifugation: 2041 ± 1078 cells/mm^2, p=0.59).

BM-MNCs isolated using density centrifugation or the filter-based method were biologically equivalent and TEVGs formed from scaffolds seeded by each method were structurally similar when examined up to 14-days post-implantation in our *in vivo* murine model.

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LIST OF ABBREVIATIONS

α -SMA – alpha smooth muscle actin
BMC – bone marrow cell
BM-MNC – bone marrow-derived mononuclear cell
cDNA – complementary deoxyribonucleic acid
cGMP- current Good Manufacturing Practice
CHD – congenital heart disease
CMV – cytomegalovirus
CT – computed tomography
Ct – cycle threshold
CTA – computed tomography angiography
DNA – deoxyribonucleic acid
ePTFE – expanded polytetrafluoroethylene
EVG – elastica van Gieson
FACS – fluorescence-activated cell sorting
FDA – Food and Drug Administration
H&E – hematoxylin and eosin
hBM-MNC – human bone marrow-derived mononuclear cell
HLA – human leukocyte antigen
HPRT – hypoxanthine phosphoribosyltransferase
INR – International Normalized Ratio
ISO – International Organization for Standardization
IVC – inferior vena cava
LRF – leukocyte reduction filter
MCP-1 – monocyte chemotactic protein 1
MMP-2 – matrix metalloproteinase-2
MRI – magnetic resonance imaging
OCT – optimal cutting temperature
P(CL/LA) – poly (ε)-caprolactone and L-lactide copolymer
PBS – phosphate-buffered solution
PET – polyethelene terephthalate (Dacron)
PGA – polyglycolic acid
PLA –polylactic acid
PLLA – poly-L-lactic acid
PTFE – polytetrafluoroethylene
qPCR – quantitative polymerase chain reaction
RNA – ribonucleic acid
RPMI-1640 – Roswell Park Memorial Institute medium

SCID/bg – scid/scid, beige/beige double-mutant mouse strain
SEM – scanning electron microgram
TEVG – tissue engineered vascular graft
VEGF – vascular endothelial growth factor
vWF – von Willebrand factor
WHO – World Health Organization
YARC – Yale Animal Resource Center

INTRODUCTION

The field of vascular tissue engineering is driven by the demand for durable vascular conduits that mirror the performance of native blood vessels. One important limitation in this process is the time required to create a viable neo-vessel. At first, tissue engineered scaffolds were seeded *in vitro* and incubated for several weeks to allow for the development of a vascular architecture prior to implantation. For the next generation of tissue engineered grafts, scaffolds were seeded and incubated *in vitro* for several hours before being implanted and allowed to transform into a viable vessel *in vivo*. In the current study, we strive to decrease the time and labor required to isolate the cells used for seeding. If this process can be streamlined appropriately then the entire process from obtaining cells to seeding the scaffold and implanting the graft into a patient can all occur during the intraoperative time frame of one operation. To understand this process, some background is required.

In the United States, congenital heart disease (CHD) affects approximately 1% of live births. Most infants born with a single functional ventricle, which include those with hypoplastic left heart syndrome, pulmonary atresia or tricuspid atresia, require surgical treatment to prevent cyanosis, volume overload, and congestive heart failure. The goal of surgical correction is to separate systemic circulation from pulmonary circulation to prevent the mixing of deoxygenated and oxygenated blood.

This correction is most often accomplished through a staged Fontan operative procedure in which systemic venous blood returning to the right heart is instead routed directly to the pulmonary artery, bypassing the single functional ventricle. Following passage through the lungs, the oxygenated blood returns to the single functional ventricle through the pulmonary veins and is pumped in normal fashion into the aorta to deliver oxygen to tissues[1]. The Fontan procedure is conducted in two separate stages. During the first stage of the Fontan procedure, the superior vena cava is connected to the pulmonary artery, thereby decreasing the workload of the single ventricle. However, these patients often display significant hypoxia because deoxygenated blood carried by the inferior vena cava (IVC) continues to return directly to the single ventricle where it mixes with oxygenated blood returning from the lungs. Therefore, most patients undergo the second stage of the Fontan procedure, which connects the IVC to the pulmonary artery and allows all deoxygenated blood to bypass the heart and flow directly to the lungs. However, because the IVC is not adjacent to the pulmonary artery, this distance is bridged by placing a vascular conduit between the IVC and the pulmonary vasculature.

Types of grafts:

Currently, grafts are most often composed of biocompatible, but synthetic, polymers including polyethylene terephthalate (PET, Dacron), expanded-polytetrafluoroethylene (ePTFE, Gore-Tex), polytetrafluoroethylene (PTFE, Teflon) and polyurethanes[2]. These synthetic polymers, however, are associated with a number of short and long-term complications that most notably include stenosis, thromboembolism, calcium deposition, and infection[3,4]. Furthermore, the 5-year patency rate in pediatric cardiac surgery for

these procedures remains between 65% and 90% while long-term follow-up demonstrated high graft failure with between 70% and 100% failing at 10-15 years[5].

In addition to synthetic grafts, biologic grafts may be used. These grafts are crafted using autologous tissues, including saphenous vein and pericardium, allografts, and xenografts[6]. Biologic grafts have lower rates of thromboembolism than synthetic grafts. However, they also have increased rates of calcification, graft failure, and pseudoaneurysm formation compared with synthetic grafts[7,8]. Most significantly, however, none of these graft material options have the ability grow with the patient. Since pediatric patients often outgrow surgically implanted grafts, patients with such a surgical scenario require reoperation[9]. Such redo procedures are reported to have significantly higher mortality and morbidity than initial sternotomies[10].

Any new graft material should address the limitations of both synthetic and biologic grafts to reduce the overall mortality and morbidity in patients with CHD and improve their postoperative quality of life. The ideal vascular graft would be easily implantable with ease of handling during surgery, have low levels of stenosis, low rates of thromboembolism, high growth potential, and be resistant to infection.

Scaffolds:

The goal of tissue engineering is to provide materials that incorporate into and function similarly to the patient's native tissue thus allowing normal physiologic function. This is accomplished using scaffolds that function as a surface for cell attachment and growth

followed by new tissue formation on the scaffold. To promote the creation of functional tissue, the scaffolds must resemble the desired tissue in both size and shape[11]. The formation of new tissue is followed by degradation of the original scaffold leaving a fully functional neo-vessel composed solely of autologous tissue. Pluri- or multipotent stem cells are often used in the process to create these tissue engineered vascular grafts (TEVGs) (Figure 1).

A successful TEVG scaffold material should have the following three characteristics: 1) be biodegradable, 2) be anti-thrombotic, and 3) have adequate porosity and pore size to allow for cell attachment[12]. The most common polymers used for tissue engineered scaffolds include variations of polyglycolic acid (PGA) and polylactic acid (PLA) used in conjunction with poly (ε)-caprolactone[13,14].

Some researchers have developed TEVGs using decellularized allogeneic human or xenogenic porcine vessels seeded with autologous endothelial cells from the recipient[15]. However, allo- or xenotransplantation carries the risk of developing an immunologic response with subsequent destruction of the graft. Choosing the correct material is essential for the creation of TEVGs that will adequately remodel into a viable neovessel. Several important factors include biocompatablity, mechanical properties, and the biodegradation profile.

It is important to note that TEVGs have rigidity. Their lumen does not collapse when implanted *in vivo* due to the biomechanical properties of the scaffolds. The tensile

strength of PGA with a 50:50 copolymer sealant solution of poly-(ε)-caprolactone and L-lactide (P(CL/LA)) is approximately twice the tensile strength of native venous tissue[16]. In addition, the suture retention strength of PGA-P(CL/LA) scaffolds are greater than the corresponding values for native veins[17]. Further, the resultant burst pressure of PGA-P(CL/LA) scaffolds under increasing pneumatic pressure is significantly greater than the physiologic burst pressure for native veins[18]. Finally, the intrinsic elasticity of PGA-P(CL/LA) scaffolds, measured using Young's modulus, indicates that these scaffolds are more elastic than synthetic ePTFE grafts but stiffer than native venous tissue[18-20]. Young's modulus is defined as the ratio of stress (force per unit area) along an axis to the strain (ratio of deformation from initial length) along the same axis[21].

TEVGs have a rich history with initial development in the early 90s of highly porous biocompatible scaffolds cultured with smooth muscle[22]. Subsequently, TEVGs constructed using autologous myofibroblasts and endothelial cells seeded *in vitro* onto PGA fiber scaffolds were surgically implanted into lambs. These were the first studies to successfully create viable vascular conduits in a large animal model[23]. In this lamb model, venous cells were harvested from explanted autologous vein and expanded in cell culture. These cultured cells were labeled with acetylated low-density lipoprotein that is selectively absorbed by endothelial cells. Following an additional 24-hours of incubation, cells were sorted into endothelial cell (low-density lipoprotein positive) or smooth muscle cell and fibroblast (low-density lipoprotein negative) populations. These two populations of cells were then seeded onto PGA scaffolds. The scaffold was maintained in culture for seven days after which the endothelial-rich population from the original cell culture was

seeded onto the inner lumen. This scaffold was maintained in culture for an additional day. When harvested six months after implantation, all seeded scaffolds remained patent and free of aneurysm formation, and histology showed significant collagen formation, elastic fiber content, and endothelialization of the lumen[23].

Scaffold seeding:

The previously described method of seeding TEVGs was successful and firmly established the feasibility of using TEVGs for the surgical treatment of congenital heart defects. However, the widespread clinical utility of this process was severely limited by the labor intensive process of obtaining the precursor cells and the prolonged time period needed to adequately expand the cells in culture before they could be used for scaffold seeding.

In search of a more efficient method of seeding biodegradable scaffolds Noishiki et al. had noted that grafts seeded with bone marrow cells (BMCs) would release autocrine molecules leading to the formation of a luminal endothelial monolayer in a canine aortic model[24]. The early development of a luminal endothelium is important because it prevents the formation of acute thrombosis and subsequent graft occlusion. However, because they had used a synthetic, non-biodegradable ePTFE scaffold in their experiment, Matsumura et al. decided to test the feasibility of creating viable TEVGs using biodegradable scaffolds seeded with the more easily obtained BMCs[25]. In this experiment, bone marrow was aspirated from the iliac crest of dogs and bone marrow-derived mononuclear cells (BM-MNCs) were isolated using density centrifugation. These

cells were seeded onto the luminal surface of a poly-L-lactic acid (PLLA)-P(CL/LA) scaffold and incubated in culture for two hours to promote cell adhesion. The scaffolds were implanted as intrathoracic IVC interposition grafts and harvested over a period of two years. For an interposition graft, a segment of the native vessel is removed and replaced with the TEVG. The two ends are reconnected (anastomosed) using sutures. Over the course of the experiment, all seeded grafts remained patent without evidence of aneurysm formation, thrombosis or stenosis. Pre-implantation analysis demonstrated retention and adherence of BM-MNCs to the scaffold and immunohistochemical analysis of explanted tissue was positive for luminal endothelial markers (von Willebrand factor (vWF), factor VIII, CD31 and CD146), medial smooth muscle markers (SM1, SM2, SMemb, and α-actin), and markers of angiogenesis (vascular endothelial growth factor (VEGF) and Ang-1)[25]. This study illustrated that autologous BM-MNCs could be used to create viable TEVGs with several benefits over using cells from explanted venous tissue. First, obtaining venous cells from explanted tissue requires an invasive procedure with numerous risks, especially wound complications such as dehiscence, prolonged drainage, cellulitis, and hematoma formation[26]. Second, cells cannot always be obtained from explanted tissue and this is affected by the patient's comorbidities and age due to the limited capacity of adult somatic cells to replicate[27]. Third, the expansion of cells from explanted tissue takes weeks and severely limits the usefulness of this method in clinical settings. Fourth, the prolonged exposure of the cells to environmental factors from the culture media, pathogens, and even autologous molecules increases the risk of contamination or cellular dedifferentiation. BM-MNCs overcome these issues by eliminating the need for invasive harvesting techniques and prolonged cell-line

expansion. This in turn limits the risk of bacterial contamination and mitigates decreases in cellular viability and function.

Clinical studies:

The first human clinical trial using TEVGs to treat children with CHD was started at Tokyo Women's Medical University in April 1999. For the first three patients in the trial, venous cells were obtained through vein harvesting, expansion in *ex vivo* cell culture and followed by incubation of the seeded scaffolds. In 2001, the method of scaffold seeding was changed first to direct bone marrow seeding and finally to seeding using BM-MNCs as based on the results described above[28-30]. Scaffolds seeded with BM-MNCs were incubated preoperatively for two to four hours before implantation. In 2005, at midterm follow-up of 42 patients who underwent surgery between 2001 and 2004, with average time since operation of 1.3 years, there were no graft related complications. This included no aneurysm formation, ectopic calcification, acute thrombosis, stenosis, or graft occlusion. While all grafts remained patent at time of analysis, the diameter of the grafts had increased on average to 110 ± 7% of their original size. One patient with hypoplastic left heart syndrome had died of complications unrelated to TEVG function 3 months following operation[27].

At long-term follow up of 25 patients with implanted grafts with average time from operation of 5.8 years, there was no graft-related mortality, graft infection, ectopic calcification, graft failure, or aneurysm formation. Three patients had died of causes unrelated to TEVG function and serial cineangiography or computed tomography (CT)

taken prior to death showed patent grafts[31]. This is in sharp contrast to outcomes in patients undergoing Fontan procedures using synthetic grafts. In a study of 193 patients undergoing extracardiac Fontan procedures, overall freedom from graft failure was 89% at five years and 85% at 10 years. At 15 years, 6.7% of surviving patients had developed thrombosis with 3% presenting with significant obstruction diagnosed during Doppler echocardiography. At 15 years, cumulative freedom from cavopulmonary pathway obstruction was 89%[3]. In a study of 200 patients at 10-year follow-up, reoperation was performed in 12% of patients and thromboembolism occurred in 6.5% of patients with 84.6% of these occurring within one year of their operation[32]. Patients in the TEVG study received three to six months of anticoagulation with warfarin and aspirin (INR- International Normalized Ratio recommended between 1.5 and 2). From six to 12 months, patients were maintained on aspirin alone after which time anticoagulation was discontinued[28]. Patients with synthetic grafts, in contrast, often remained on anti-aggregation therapy indefinitely[3,33].

TEVG growth potential:

As discussed earlier, one of the main advantages of using TEVGs in the pediatric population is their ability to grow with the child. To characterize and evaluate the growth potential of TEVGs, Brennan et al. implanted PGA grafts into a juvenile lamb model[34]. It is important to note that each animal model has specific characteristics that make it ideal for the evaluation of certain biological processes. In pediatric patients undergoing corrective surgery for congenital heart disease, ectopic calcification is a leading cause of graft failure[23]. Importantly, lamb vasculature undergoes accelerated ectopic calcification

during growth as compared to other large animal models such as pigs and dogs[35]. The juvenile lamb study demonstrated patent TEVGs at six months with no evidence of rupture, thromboembolism or significant ectopic calcification. Serial magnetic resonance imaging (MRI) showed an increase in TEVG size proportional to the unaltered right pulmonary artery (control). Quantitative analysis revealed the volume of TEVGs at six months averaged 126.9 ± 9.9% of their volume at one month and that wall thickness was comparable between the TEVG and native IVC (control). At six months, histological analysis demonstrated that the implanted TEVGs and native vein had comparable amounts of elastin, collagen and glycosaminoglycan. Immunohistochemistry revealed endothelialization of the lumen with surrounding layers of smooth muscle cells. Importantly, this was the first study to demonstrate expression of Eph-B4 in TEVGs, which is a marker of venous differentiation expressed during native venous tissue growth[34]. This finding suggests that TEVG remodeling resembles normal venous development.

Promising results from the aforementioned studies and improvements in the efficiency of creating and seeding TEVGs led to the 2009 Food and Drug Administration (FDA) approval at Yale of a US clinical trial of TEVGs in pediatric patients with single ventricle cardiac anomalies.

Small animal models:

Large animal models are an essential step in providing important data on the safety and efficacy of TEVGs that can be readily translated to human clinical trials. However, these

models are less ideal for analyzing the molecular and cellular mechanisms responsible for graft remodeling and for elucidating the processes involved in the development of neovessels. Mice are an ideal organism for studying the biologic development of cardiovascular tissue due to their short generation time, accelerated lifespan, and the homology between human and murine genes responsible for numerous complex genetic pathologies such as atherosclerosis[36,37]. One major limitation for using a murine model was developing a functional scaffold with a sub-1 mm internal diameter that could then be implanted using microsurgical techniques. The development of the first small-diameter biodegradable scaffold was reported in 2008 by Roh et al[18]. They developed a dual cylinder system that allowed nonwoven felts of PGA to be shaped into tubes during insertion into the dual cylinder chamber. Next, 21 gauge stainless steel rods were introduced into the lumen of the scaffolds before application of a 50:50 copolymer sealant solution of P(CL/LA) to prevent collapse of the scaffold inner lumen. The resulting tubes were rapidly frozen to transform the P(CL/LA) sealant from the liquid to the solid phase leading to a solid, non-collapsing porous scaffold. Scanning electron microgram (SEM) revealed an internal diameter of 0.9 mm with a wall thickness of 150 μm[18]. Of note, current synthetic materials such as PTFE or PET (Dacron) cannot be used to create clinically functional grafts with sub-1 mm internal diameters[38].

Density centrifugation versus filter collection:

At this juncture, our group had developed viable animal models to characterize the molecular development of neo-vessels and had shown the effectiveness of BM-MNCs in this process numerous times. However, several important limitations remained as

obstacles to the widespread clinical application of TEVGs. Currently, BM-MNCs are generally isolated from bone marrow using density centrifugation with Ficoll, a soluble high-mass polysaccharide used to facilitate the separation of blood into its components[39]. Lower density mononuclear cells and platelets collect on top of the Ficoll layer, while higher density erythrocytes and granulocytes collect below the Ficoll layer. Subsequent centrifugation and washing of the top layer with phosphate-buffered saline (PBS) can be used to separate mononuclear cells from platelets[40]. There are, however, several drawbacks to using Ficoll density gradient centrifugation to isolate BM-MNCs. First, erythrocytes may form aggregates with mononuclear cells causing unwanted sedimentation of the mononuclear cells into the bottom layer. Second, repeated and prolonged handling of the sample may decrease viability of isolated cells. Third, cellular damage may cause cell aggregation leading to decreased yield of mononuclear cells[41]. Fourth, density centrifugation is affected by operator proficiency leading to significant variability[42]. Fifth, Ficoll isolation uses an open method that exposes the sample to the environment during isolation. In clinical practice, an open system must use an International Organization for Standardization (ISO) class 7 clean room to limit the risk of bacterial contamination and meet the standard of current Good Manufacturing Practice (cGMP). These clean rooms are expensive to construct and maintain in compliance with sterile production standards. They also require substantial human resources to operate[43].

If a sterile disposable blood filter could be integrated into a closed system of collection, it would not require a clean room, would be operator-independent, would decrease the risk of contamination, would reduce cellular damage and facilitate the clinical use of TEVGs.

The World Health Organization (WHO) defines a closed system as the aseptic collection and separation of blood components under clean conditions sealed from the external environment[44]. In this system, bone marrow can be transferred directly into a sterile plastic holding chamber using a syringe. The bone marrow is filtered by gravity-mediated downward longitudinal flow and mononuclear cells are captured on a nonwoven polyester fiber filter media by interception. The trapped BM-MNCs are recovered by back-flushing the filter and reversing the direction of flow through the closed system. This filter was originally adapted from leukocyte reduction filter-based systems (LRF) used by blood banks to facilitate the isolation of white blood cells from blood products. Use of this blood filter in clinical transfusion practice helps expedite the removal of some cell associated viruses (CMV-cytomegalovirus) and decrease human leukocyte antigen (HLA) alloimmunization, which in turn decrease transfusion reactions, infections and febrile episodes[45].

The ability of a filter-based method to effectively isolate mononuclear cells was illustrated by Hibino et al when they successfully isolated MNCs from human bone marrow (hBM-MNCs)[46]. They subsequently seeded PGA-P(CL/LA) with hBM-MNCs isolated using either the traditional density centrifugation with Ficoll method or using the experimental filter-based method. These seeded scaffolds were implanted as IVC interposition grafts in immunocompromised SCID/bg mice. These mice have impaired lymphoid development and reduced natural killer cell activity which prevents host rejection of the implanted human cells[47]. It took significantly less time for cell isolation by filtration (10 ± 12 min) compared to density centrifugation (106 ± 11 min). Despite

this, there was no significant difference in the number of hBM-MNCs isolated by either method. Fluorescence-activated cell sorting (FACS) showed that the percentage of viable cells and the ratio of stem cells to mononuclear cells were comparable between both groups. Analysis of TEVG histology at 10 weeks post-implantation revealed no difference between scaffolds seeded by either method. Both graft groups demonstrated an endothelial monolayer as evidenced by positive luminal vWF staining. Positive alpha smooth muscle actin (α-SMA) and calponin staining demonstrated the formation of a smooth muscle layer in both groups. Serial monitoring by ultrasonography, CT angiography (CTA) and post-explant analysis revealed no aneurysm formation or graft rupture over 10 weeks in both groups.

This technique was subsequently used to create TEVGs that were effectively implanted into a large animal model[48]. Specifically, bone marrow was obtained from lambs and BM-MNCs were isolated using an open system density centrifugation method or a closed system filter-based method. PGA-P(CL/LA) scaffolds were seeded with cells isolated using either method and implanted into juvenile lambs as intrathoracic IVC interposition grafts. Results demonstrated that total procedure time from removal of bone marrow to insertion of graft was significantly less using the closed system (2 hrs 17 min) compared to the open method (4 hrs 28 min). Further, there was no significant difference in the number of cells seeded onto scaffolds in either group. Two animals in the open method group developed significant graft stenosis requiring early sacrifice, while all six lambs in the closed method group survived without any evidence of graft related complications including acute thrombosis, stenosis, or aneurysm formation. Histologic analysis of tissue

explanted six months after implantation demonstrated that the cellular architecture in both groups mirrored native vein (control) with the development of three distinct layers including an intimia, media and adventitia[48].

Current thesis research:

At this stage, it had been shown that scaffolds seeded with BM-MNCs isolated via a closed filter-based system could develop viable TEVGs that resembled native tissue in immunocompromised mice[46]. However, we needed to show that this was possible in immunocompetent mice. Therefore, to compare the effectiveness of the novel filter-based collection method with the conventional density centrifugation technique using Ficoll, we isolated BM-MNCs from the bone marrow of wild type, immunocompetent C57BL/6 mice using the two methods. Then we seeded biodegradable scaffolds with the isolated BM-MNCs and implanted them as IVC interposition grafts into the same strain of mice. We hypothesized that neo-tissue formed from the biodegradable scaffolds seeded using BM-MNCs isolated from each method would be biologically, structurally, and functionally equivalent.

METHODS AND MATERIALS

Scaffolds:

A 50:50 fiber mesh constructed with a poly-glycolic acid coated with copolymer sealant solution of poly-L-lactide-co-ε-caprolactone was used for scaffold construction[18,49]. Scaffolds were 4 mm long with a diameter of 0.9 mm.

Bone marrow-mononuclear cell preparation and scaffold seeding:

BM-MNCs were extracted from the bone marrow of immunocompetent CB57BL/6 wild type mice through two distinct methods, specifically, density centrifugation using Ficoll versus filtration. For the filtration method, BM-MNCs were isolated from bone marrow as follows: 5 mL of bone marrow was extracted from a minimum of five mice and the resulting volume was increased to 15 mL by adding RPMI 1640. Using a syringe, this mixture was transferred to a sterile storage chamber that was connected to a scaled down version of the commercially available polyester fiber human blood cell filter. This filter functions to capture BM-MNCs in the filter media through interception, thus retaining the needed cells. The filters were washed twice with PBS to remove entrapped and unwanted erythrocytes from the filter. The BM-MNCs that were retained in the filter were isolated by reverse-flushing the filter with 6 mL of 10% dextran 40/saline solution. This solution was centrifuged and the resulting pellet was diluted using RPMI-1640 (Figure 2)[48]. For Ficoll density centrifugation, bone marrow was extracted and centrifuged at 400 g for 30 min in Histopaque-1083. The low density layer containing monocytes was removed and transferred into a new tube. This was washed with PBS and centrifuged at 100 g for 10

min. The supernatant was removed and fresh PBS was added to the resulting pellet. This pellet was centrifuged for a third time at 100 g for 10 min. The supernatant was again removed and the isolated BM-MNCs were suspended in RPMI 1640[49]. For scaffold seeding, $1.0x10^6$ BM-MNCs isolated using either method were seeded onto the luminal graft surface manually using a pipet. The seeded scaffolds were placed in 1 mL of sterile RPMI-1640 in a CO_2 incubator for 24 hours at 37 ^oC to promote adherence of BM-MNCs to the scaffold[49].

Seeded scaffold cell counts:

Following BM-MNC isolation using the density centrifugation and filter methods, manual cell counts were performed for each group. In addition, cell viability was assessed using trypan blue staining also followed by manual cell counting. The deoxyribonucleic acid (DNA) content of seeded scaffolds following 24 hour incubation was obtained using a PicoGreen DNA detection assay[42].

Surgical implantation into murine model:

Animals were treated appropriately as required by the National Institutes of Health Guide for the Care of, and the Yale University policy on, Use of Laboratory Animals. The Yale Institutional Animal Care and Use Committee approved the use of animals and procedures for this study. All mice were 10-week-old ‘wild type’ C57BL/6 mice purchased from Jackson Laboratories.

TEVGs were implanted as IVC grafts by members of our lab in CB57BL/6 mice with n = 23 for each group, group 1 (density centrifugation) and group 2 (filter), using standard microsurgical technique as follows: All mice were anesthetized with an intraperitoneal injection of 0.10 ketamine/xylazine mixture. A midline laparotomy incision from the xyphoid to the suprapubic region was made and the intestines were wrapped in saline-moistened gauze to approach the inferior vena cava. Microvascular clamps were used to clamp the IVC followed by transection of the vessel. The interposition graft was introduced and secured using end-to-end anastomoses with sutures. Heparinized solution was used frequently to prevent formation of an acute thrombosis[18,49]. Mice were kept in regularly cleaned and maintained cages by the Yale Animal Resource Center (YARC) where they were fed *ad libitum*. All mice were sacrificed 14-days following implantation and grafts were explanted following saline perfusion of the circulatory system.

Ultrasound monitoring of implanted TEVGs:

At 3, 7, and 14-days post-implantation graft luminal diameter was determined using ultrasonography and graft patency was determined by assessing flow velocity both proximal and distal to the graft using Doppler ultrasonography. To perform ultrasonography on mice, 1.5% isoflurane was used as an anesthetic.

Histology:

Following graft explantation at 14-days, grafts were fixed using 4% para-formaldehyde, embedded in paraffin, sliced into 5 μm sections, and mounted onto slides. These sections were stained using Hematoxylin and Eosin (H&E), Alcian Blue (mucins), von Kossa

(calcium), Masson's trichrome (collagen), and Elastica van Gieson (EVG) (elastin). On H&E stained specimens, the intima, media, and adventitia were identified and measured manually using ZEN lite, and post-explantation graft patency was defined as a luminal diameter greater that 50% compared to the graft diameter prior to implantation.

Immunohistochemistry:

Paraffin embedded sections of explanted graft, created as described above, were stained with anti-vWF, anti-SMA, anti-matrix metalloproteinase-2 (MMP-2) and anti-F4/80 antibodies to identify murine endothelial cells, smooth muscle cells, MMP-2 and macrophages respectively. Anti-iNOS and anti-CD206 antibodies were used to identify M1 and M2 macrophage phenotypes respectively. Biotinylated IgG was used to detect primary antibody attachment. This was followed by streptavidin-horse radish peroxidase binding and color development, which was accomplished using 3,3-diaminobenzidine.

Macrophage quantification:

Macrophages were identified using F4/80 expression as described above. Each stained graft section was dived into eight regions and stained macrophage nuclei were counted manually in three of the eight regions at 400x magnification.

Ribonucleic acid (RNA) extraction real time-quantitative polymerase chain reaction:

Grafts explanted following 14-days of implantation were embedded in optimal cutting temperature (OCT) compound and rapidly frozen in a -80 ^{o}C freezer. These frozen grafts were sliced into twenty 30 μm sections and total RNA was extracted and purified using a

Qiagen RNeasy mini kit according to the manufacturer's instructions. Reverse transcription was performed using an Applied Biosystems high capacity RNA-to-cDNA kit. Quantitative polymerase chain reaction (qPCR) was performed using a Step One Plus Real-Time PCR system using a TaqMan Universal PCR Master mix Kit with the following primers and reference numbers: CCR2 (Mm00438270_m1), itgam (Mm00434455_m1), ym1 (Mm00657889_mH), and HPRT (Mm00446968_m1) as a control. In real-time qPCR, gene replication is detected by an increase in fluorescent signaling and cycle threshold (Ct) is defined by the number of replication cycles required to surpass a predefined threshold (which is based on a background level of gene expression). Therefore, Ct levels are inversely proportional to the amount of target messenger RNA in the sample. Relative quantification compares the change in expression of the target gene in the TEVG from native IVC in relation to the change in expression of an endogenous reference gene, hypoxanthine phosphoribosyltransferase (HPRT), between the TEVG and native IVC. This is referred to as the comparative cycle threshold method and results as reported as ΔΔ Ct which describes the change in expression of the target gene in the TEVG compared to expression in control native IVC[50].

Statistical analysis:

Based on results by Hibino et al. in 2011, a patency rate of 70% in grafts seeded with density centrifugation isolated BM-MNCs versus 30% in unseeded grafts at 14-days post-implantation was assumed[51]. Based on these data, sample size was calculated using Fisher's exact probability test using an alpha-error of 0.05 and a power of 0.8.

All numerical values are listed as the mean ± 1 standard deviation and the sample size is also included. Statistical significance was determined as a P value < 0.05. The Student's *t* test was used for continuous variables with normal distribution while the Welch's *t* test was used for two groups with unequal variance (substantially different standard deviations). Continuous variables with non-normal distribution were evaluated using the nonparametric Mann-Whitney test. Dichotomous variables were evaluated using the Fisher's exact test.

Procedure	Involvement
Scaffold construction and seeding	Primary
BM-MNC isolation	Primary
Seeded scaffold cell counting	Primary
Surgical implantation of scaffolds	Assistant to Tai Yi and Hirotsugu Kurobe
TEVG ultrasound monitoring	Primary
Histology preparation	Yale Histology and Histomorphometry Laboratory
Histology analysis	Primary
Immunohistochemistry	Primary
Macrophage quantification	Primary
RNA extraction and RT-qPCR	Primary
Statistical analysis	Primary

Table 1. Involvement with specific procedures conducted during the course of this experiment. The author had primary involvement with all aspects of the experiment except surgical implantation of scaffolds in the murine model and with preparation of histology slides.

RESULTS

Comparison of BM-MNC isolation:

Both total BM-MNC and viable BM-MNC counts following filtration were significantly greater than those seen following density centrifugation-based cell isolation (total cells, filter: 44.3 ± 12.6x10^6 cells/mouse versus density centrifugation: 24.8 ± 8.8x10^6 cells/mouse, p=0.02; viable cells, filter: 32.8 ± 6.7x106 cells/mouse versus density centrifugation: 20.6 ± 8.7x106 cells/mouse, p=0.04; Figure 3). The filter-based BM-MNC isolation method also showed significantly greater cell attachment following 24-hour scaffold incubation as detected by PicoGreen DNA assay evaluation of scaffold DNA content between the two groups (filter: 15.5 ± 6.3x10^3/mm^2 versus density centrifugation: 12.4 ± 2.5x10^3/mm^2, p=0.04; Figure 4).

TEVG monitoring via ultrasound:

Serial ultrasonographic evaluation on day 3, 7, and 14 showed no significant difference in luminal diameter of graft patency between the filtration and density centrifugation methods. In both groups, the graft patency and the luminal diameter decreased with each time point. However, no aneurysm formation, hemorrhagic complications, thrombus formation or embolization was detected in either group.

Evaluation of graft patency and luminal diameter:

There was no statistically significant difference in graft patency between the two groups as determined using H&E stained slides of grafts explanted following 2 weeks of

implantation (filter: 78% patency versus density centrifugation: 87% patency, p=0.7; Figure 5A). Manual measurement of patent grafts did not show any statistically significant difference in neointimal thickness (filter: 37.9 ± 11.2 µm versus density centrifugation: 37.9 ± 7.8 µm, p=0.99) or lumen diameter (filter: 633 ± 131 µm versus density centrifugation: 620 ± 82.9 µm, p=0.72) (Figure 5B).

TEVG histology:

Neovessel formation, cellular infiltration into the TEVG, cellular distribution in the TEVG, and cellular architecture appeared similar between the two groups (filter and density centrifugation) using H&E staining of grafts explanted after 2 weeks of implantation. Both groups demonstrated abundant collagen deposition within TEVGs as evidenced by Alcian blue and Masson's trichrome extracellular matrix stains. Elastica van Gieson stain demonstrated a paucity of elastin in both groups, while the absence of von Kossa staining demonstrated lack of graft calcification in either group. Of note, unabsorbed poly-glycolic acid fibers resulted in non-specific staining by both von Kossa and Alcian blue stain, and they resemble capillaries or vacuoles on H&E stain.

TEVG immunohistochemistry:

SMA immunohistochemical staining of smooth muscle cells was found mainly in the media of explanted TEVGs of both groups. vWF staining was used to identify the luminal intima of TEVGs in each group and it demonstrated endothelialization of the explanted tissue. Matrix metalloproteinase-2 is a central component for effective remodeling of seeded scaffolds into neovessels and previous studies have shown that

MMP-2 activity is greatest two weeks post-implantation in mice[52]. In our results, MMP-2 staining showed similar activity in grafts explanted from both groups at 14 days post-implantation.

TEVG macrophage analysis:

Abundant F4/80 immunohistochemical staining in TEVGs demonstrated that infiltration by macrophages occurred in both groups. There did not appear to be any difference in infiltration by the two major macrophage phenotypes, M1 and M2, in TEVGs seeded using either method of cell isolation. There was no statistically significant difference in quantitative macrophage infiltration between the number of F4/80 positive cells between the two groups (filter: 1887 ± 907.7 cells/mm^2 versus density centrifugation: 2041 ± 1078 cells/mm^2, p=0.59; Figure 6). Variation in macrophage phenotype, M1 (filter: 0.82 ± 0.67 versus density centrifugation: 1.00 ± 0.99, p=0.67) and M2 (filter: 1.26 ± 1.30 versus density centrifugation: 0.92 ± 0.67, p=0.52) , between the two groups was assessed using qPCR in explanted grafts and no statistically significant difference was found (Figure 7). Macrophage gene expression as determined by qPCR using the monocyte marker CD11b demonstrated no statistical significance between the two groups at the following time points: following isolation but prior to seeding (filter: 1.38 ± 0.68 versus density centrifugation: 1.00 ± 0.60, p=0.16) and following explantation at 2 weeks (filter: 1.03 ± 0.71 versus density centrifugation: 1.00 ± 0.55, p=0.97) (Figure 7).

DISCUSSION

The goal of our study was to assess the difference between BM-MNCs isolated using a conventional density centrifugation in Ficoll method versus a unique filter-based method. Furthermore, we analyzed the effect of each method on neo-vessel formation and remodeling of implanted scaffolds, using an immunocompetent murine model.

We found no difference in luminal diameter, graft patency, or incidence of stenosis between grafts created using scaffolds seeded by either method. There was also no difference between the histologic or immunohistochemical structure of neo-tissue with regard to intima, media, and adventitia formation, location of collagen deposition, endothelial cell attachment, smooth muscle infiltration or graft calcification. A previous study had shown differences in macrophage phenotype in isolated BM-MNCs based on method of bone marrow filtration[46]. However, we found no difference in macrophage phenotype both following bone marrow isolation and in grafts, explanted after two weeks of implantation. Further, macrophage polarization and amount of infiltration was similar in grafts seeded by either method.

These findings are important because host-initiated macrophage infiltration into the implanted TEVG is essential for vascular neo-tissue formation. In 2011, Hibino et al. analyzed the role of host macrophages in the development of TEVG stenosis[51]. When PLLA-P(CL/LA) scaffolds, unseeded or seeded were implanted as infrarenal IVC interposition grafts in CB57BL/6 wild type mice, the amount of macrophage infiltration

was directly related to the extent of graft stenosis at 14-days, but inversely proportional to BM-MNC seeding. It is important to note that macrophages are a heterogeneous subset of mononuclear cells that are an active part of a host immune response to foreign implanted grafts[53]. In an effort to characterize the function of the varying types of macrophages, they have been divided into phenotypes based on their biological function, surface markers, and cytokine profile[54]. These macrophages are often referred to as M1 or M2 cells. M1 macrophages are pro-inflammatory, cytotoxic cells that promote pathogen killing and chronic inflammation while M2 macrophages are anti-inflammatory cells that promote tissue remodeling, tissue repair and immunoregulation[55]. The 2011 study illustrated that seeded scaffolds express significantly less pro-inflammatory M1 phenotype macrophages than unseeded scaffolds[51]. It also showed that macrophages infiltrating patent grafts shift towards the M2 phenotype while those found in stenotic grafts show increased expression of the M1 phenotype suggesting that scaffold seeding decreased rates of stenosis by modulating the macrophage response[51,56]. In the same study, TEVGs were implanted into mice that were macrophage-depleted following the application of clodronate liposomes, which cause clodronate-induced apoptosis following endocytosis by macrophages[51,57]. These mice had grafts with reduced cellularity, decreased concentration of DNA per scaffold, absence of luminal endothelial cells as seen by a lack of vWF staining, absence of smooth muscle cells as evidenced by a lack of SMA staining, and decreased collagen. These findings suggest that inhibiting macrophage infiltration into implanted grafts prevents the formation of vascular neotissue and attest to the importance of macrophage involvement in neo-vessel development. To certify that this finding was not caused directly by clodronate liposome treatment,

TEVGs were implanted as infrarenal IVC interposition grafts in CD11b-diptheria toxin receptor knockout mice that were depleted of macrophages by intraperitoneal injection of diphtheria toxin. These mice exhibited diminished F4/80 staining, and absent vWF, SMA and collagen staining. Together these results suggest that lack of macrophage infiltration prevents adequate neo-vessel formation in TEVGs[51].

At this stage our lab had determined the importance of host macrophage infiltration for neotissue formation and the role of seeded mononuclear cells in recruiting macrophages. However, the exact molecular mechanism responsible for this recruitment remained elusive. To determine which molecules may be responsible for early monocyte (macrophage precursor) recruitment, the cytokine profile of scaffolds seeded with hBMCs was examined[56]. While there was a significant increase in the production of multiple cytokines, there were particularly high levels of monocyte chemotactic protein 1 (MCP-1). In 2000, Salcedo et al. had shown the importance of MCP-1 in angiogenesis, especially for the chemotaxis of human endothelial cells and an associated inflammatory response composed primarily of monocytes[58]. To investigate the isolated effect of MCP-1, biodegradable alginate microparticles were constructed and used to encapsulate recombinant human MCP-1[56]. These microparticles were embedded into scaffolds and implanted as IVC interposition grafts in SCID/bg mice where they released MCP-1 over a span of 72 hours. At one week post-implantation, monocyte recruitment was significantly greater in MCP-1 eluting scaffolds (200 ± 60 monocytes/hpf) compared to unseeded scaffolds (60 ± 12 monocytes/hpf). At 10 weeks following implantation, all MCP-1 eluting scaffolds remained patent and histologic analysis showed a monolayer of

luminal endothelial cells surrounded by smooth muscle cells and abundant collagen deposition in a pattern identical to that seen in scaffolds seeded with BM-MNCs[56].

In a parallel study, Mirensky et al. examined the importance of monocytes in graft formation[59]. PGA-P(CL/LA) scaffolds were seeded with 1) heterogeneous BM-MNC, 2) BM-MNC minus $CD14^{+}/CD45^{+}$ monocytes, or 3) isolated $CD14^{+}/CD45^{+}$ monocytes and implanted as infrarenal IVC interposition grafts in immunocompromised SCID/bg mice. When grafts were explanted at six months post-implantation, internal diameters were significantly greater in scaffolds seeded with only monocytes (1.022 ± 0.155 mm) compared to scaffolds seeded without monocytes (0.771 ± 0.121 mm)[59]. These differences allude to the importance of monocytes in the maintenance of long-term graft patency through the reduction of graft stenosis.

The results of the current study are important for several reasons. First, the presently accepted methods of BM-MNC isolation rely on density centrifugation, which is labor and resource intensive, time-consuming, and has significant variability based on operator technique[42]. A filter-based isolation method, similar to the one used in this study, was first used to isolate mononuclear cells from peripheral human blood where it was extremely effective[60]. Then it was successfully used to isolate BM-MNCs from human bone marrow and demonstrate that scaffolds seeded with either the filter-based method or density centrifugation would develop morphologically equivalent neo-vessels when implanted as IVC interposition grafts in immune-deficient SCID/bg mice[46]. As discussed earlier, however, elements of the host-mediated immune response, specifically infiltrating

macrophages, are essential for development of a neo-vessel resembling native tissue. In the present study, autologous BM-MNCs were isolated from wild type mice using filter-based versus density centrifugation methods and then implanted into immune-competent CB57BL/6 mice. This difference is significant because the host immune system is intact in these mice and it plays an integral role in the development of endothelial cells, smooth muscle cells, and collagen in neo-vessels[61,62]. Our results show that filter-based collection works just as well in an immunocompetent model as in an immunocompromised model. These results provide small animal model data that supports the safe and efficacious use for this vascular graft system in human clinical studies.

CONCLUSION

In this study a significantly greater number of total cells were isolated using the filter-based method suggesting it is more efficient than density centrifugation at BM-MNCs isolation. Further, the number of viable cells following filtration and cellular attachment measured by DNA content after 24 hour incubation of seeded scaffolds suggest the filter-based method is less destructive and damaging to cells than density centrifugation.

These results, as compared with the Ficoll density method of MNC isolation, show no difference in the biological activity of cells isolated by the filter-based method and no difference in the TEVGs created from scaffolds subsequently seeded by these cells. Importantly, the equivalence of the safety and efficacy of the filter versus density centrifugation based methods of TEVG formation, has been demonstrated in a large animal model[48]. Based on the results of this and previous studies, the filter-based method of BM-MNC isolation from bone marrow for scaffold seeding and the composition of the tissue in resulting TEVGs can be considered as biologically and structurally similar to TEVGs formed via the original density centrifugation based method. Since the filter-based method can be fabricated as a closed, sterile, and disposable system that is effective for scaffold seeding and TEVG formation, it can be utilized commercially as a method that significantly reduces cost, risk of infection, time required for cell isolation and removes operator variability. It is important to note that although a manual pipet was used for scaffold seeding in this study, a fully closed system would require vacuum seeding method as previously described[42]. The efficacy of the filter-based method has

now been established in small and large animal studies using both murine and human BM-MNCs. As the next step, process improvement studies of the filter-based method should be conducted in human patients where it has the potential to significantly reduce time under anesthesia and time in the operating room while the MNCs are collected and processed. This would substantially reduce both cost and potential patient complications from prolonged anesthesia exposure and an extended time of surgery. Further, the filter-based method would allow these operations to be performed at many more hospitals because ISO class 7 clean rooms would no longer be required. Even in large academic centers with established clean rooms, these facilities are often not in close proximity to operating rooms and bone marrow may need to be transported between buildings while an anesthetized patient is waiting on the operating table. This is especially concerning for pediatric patients. With the ability to extract bone marrow, isolate cells for seeding, seed the scaffold and implant the graft all within the operating room, the chance of mixing samples between patients is essentially eliminated. These improvements would expand the number of patients able to safely and quickly undergo procedures involving TEVG implantation thus leading to a substantial cost savings and advance in surgical care.

FIGURES

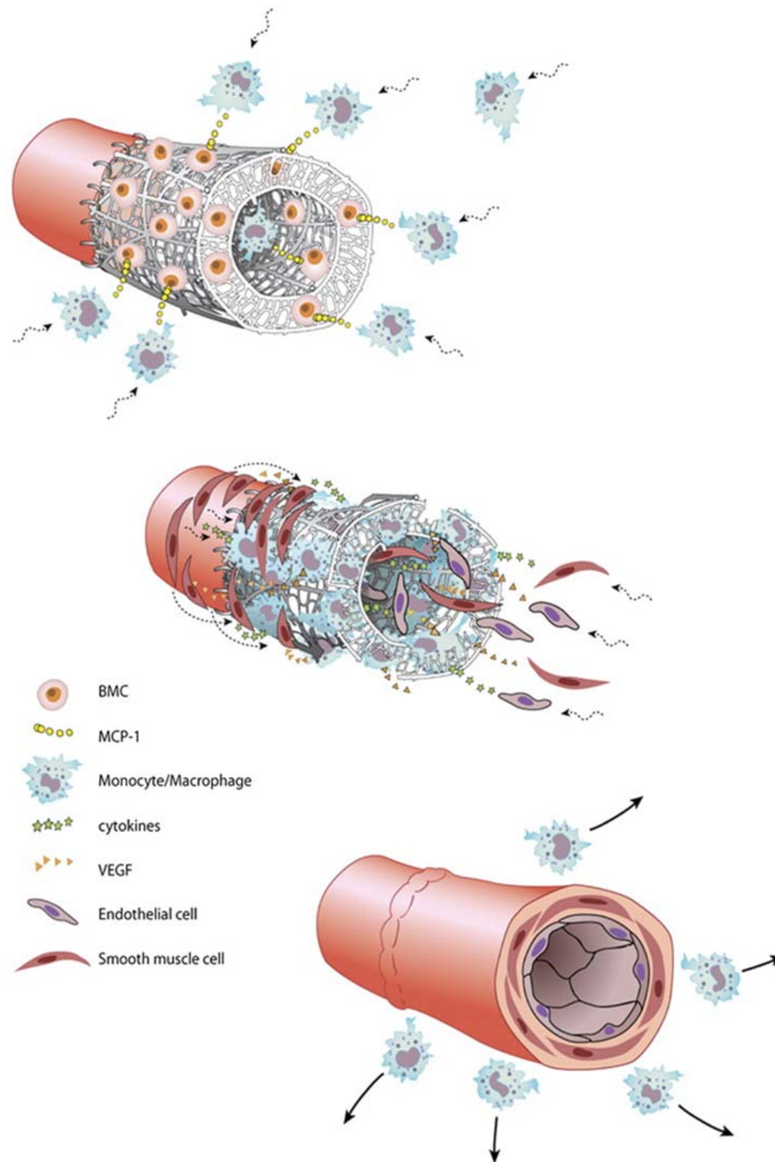

Figure 1. Neo-vessel formation from bone marrow-derived mononuclear cell (BM-MNC) seeded biodegradable scaffolds. Seeded BM-MNCs secrete monocyte chemotactic protein 1 (MCP-1) leading to monocyte infiltration. Incoming monocytes release cytokines that promote the influx of endothelial and smooth muscle cells from adjacent native vessel segments. These incoming cells create a neotissue on the luminal surface of the scaffold that resembles native tissue. The original monocytes exit the graft as the scaffold degrades leaving behind a complete neo-vessel. Reproduced from Roh et al., Ref 56, with permission from PNAS.

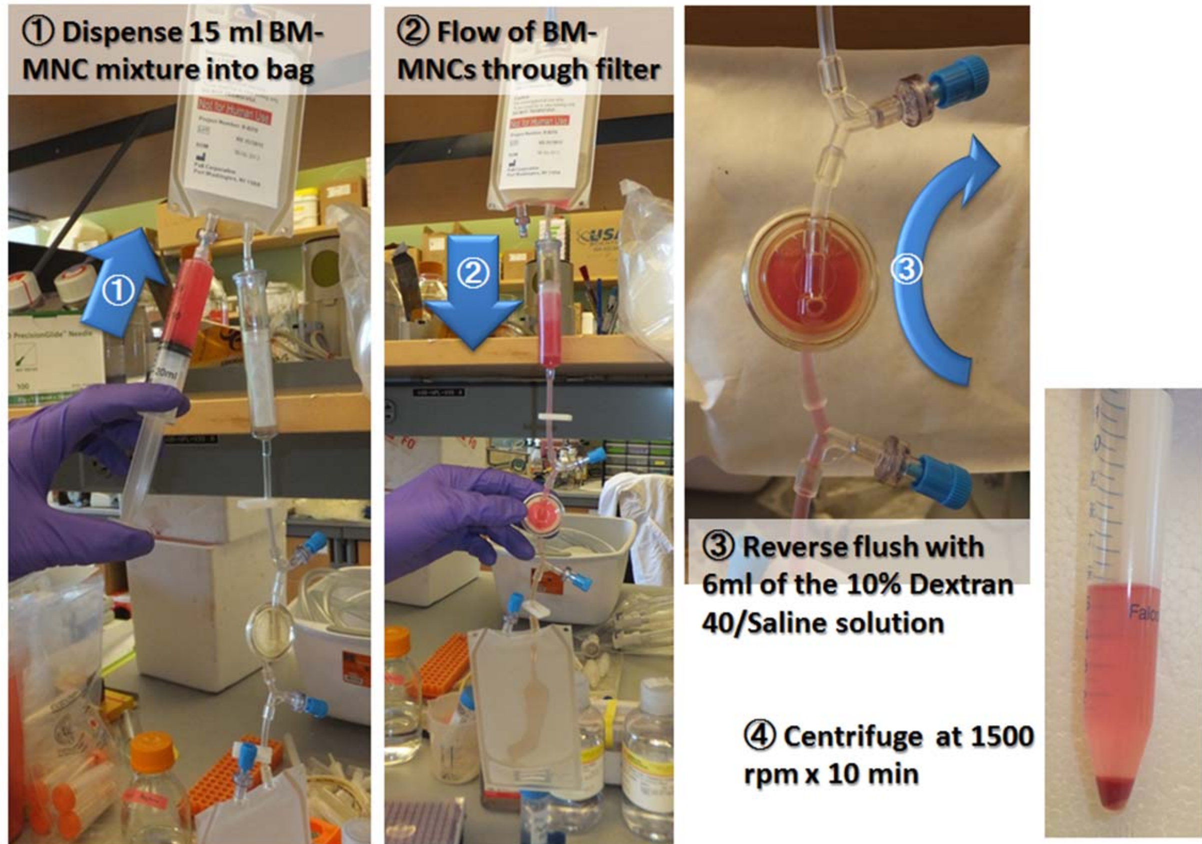

Figure 2. Closed disposable filter-based seeding system. (1) A 15 mL mixture of extracted bone marrow plus RPMI-1640 is injected into the elevated sterile bone marrow bag. (2) The mixture is passed downward through the filter media by gravitational flow, which entraps bone marrow-derived mononuclear cells (BM-MNCs) through interception. (3) Retained BM-MNCs are recovered from the filter media by reverse flushing 6 mL of harvest solution (10% dextran 40/saline) through the filter. (4) The retrieved solution is centrifuged at 1500 rpm for 10 minutes. Figure from Breuer lab.

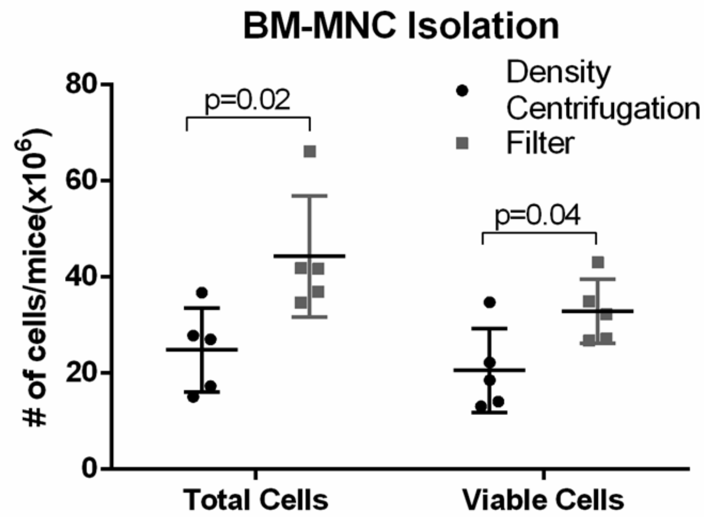

Figure 3. Evaluation of bone marrow-derived mononuclear cells (BM-MNCs) following isolation. Manual cell count was used to determine number of total BM-MNCs and trypan blue stain was used to define viable BM-MNCs. The filter-based isolation group had significantly greater total and viable BM-MNCs than the density centrifugation group following evaluation by the Student's *t* test.

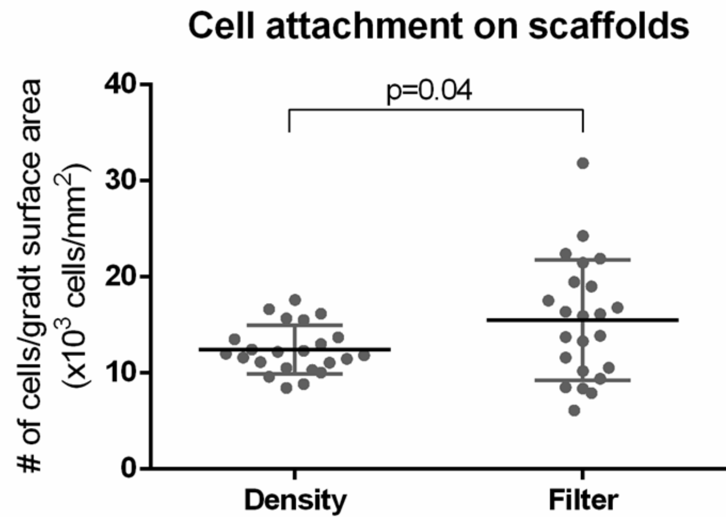

Figure 4. Cell attachment to scaffolds following seeding and incubation. DNA quantification was utilized to determine BM-MNC attachment to scaffolds following seeding and 24-hour incubation. The DNA content, used as a measurement of cell attachment, in the filter group was significantly greater than in the density centrifugation group using Welch's *t* test.

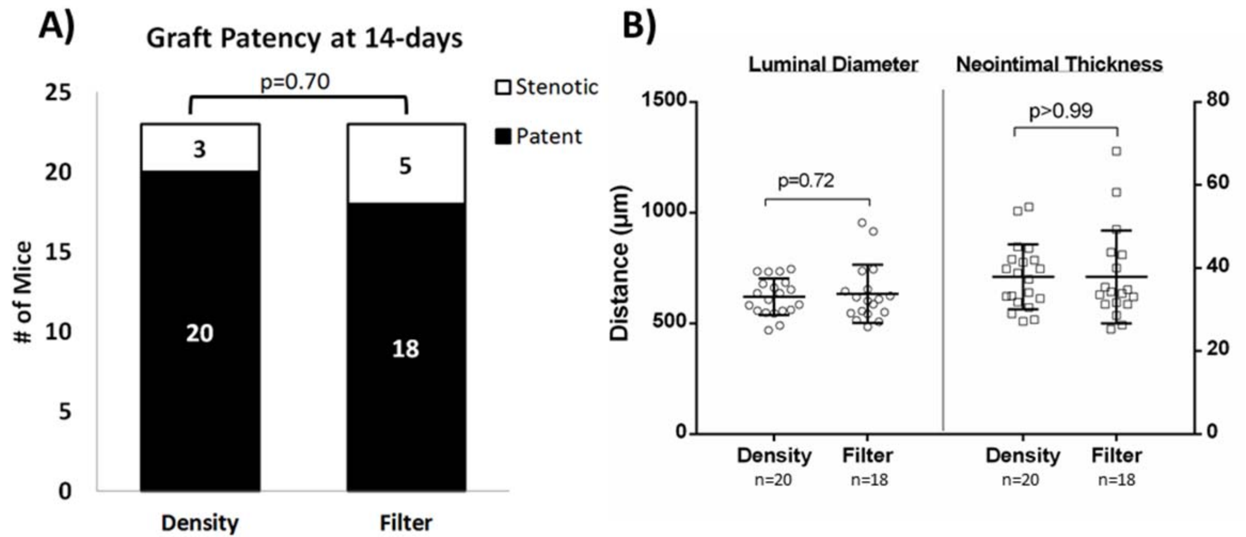

Figure 5. Analysis of TEVG structural parameters. A) Graft patency was defined as a luminal diameter greater than 50% compared to the pre-implantation graft. There was no significant difference in graft patency between the filter and density centrifugation groups according to data analyzed using the Fisher's exact test. **B)** Luminal diameter and neointimal thickness did not differ significantly between TEVGs constructed from either group following evaluation using the Welch's *t* test.

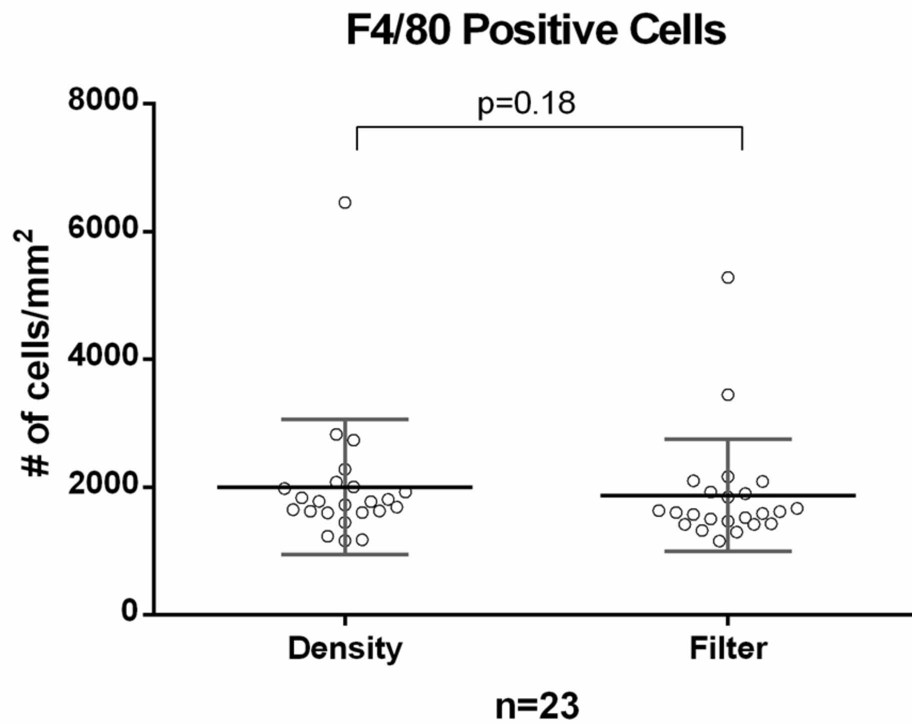

Figure 6. Macrophage infiltration in TEVGs. Macrophages were counted manually after staining for F4/80 positive cells in grafts 2 weeks after implantation. There was no significant difference between the two groups following analysis of data using the Mann-Whitney test.

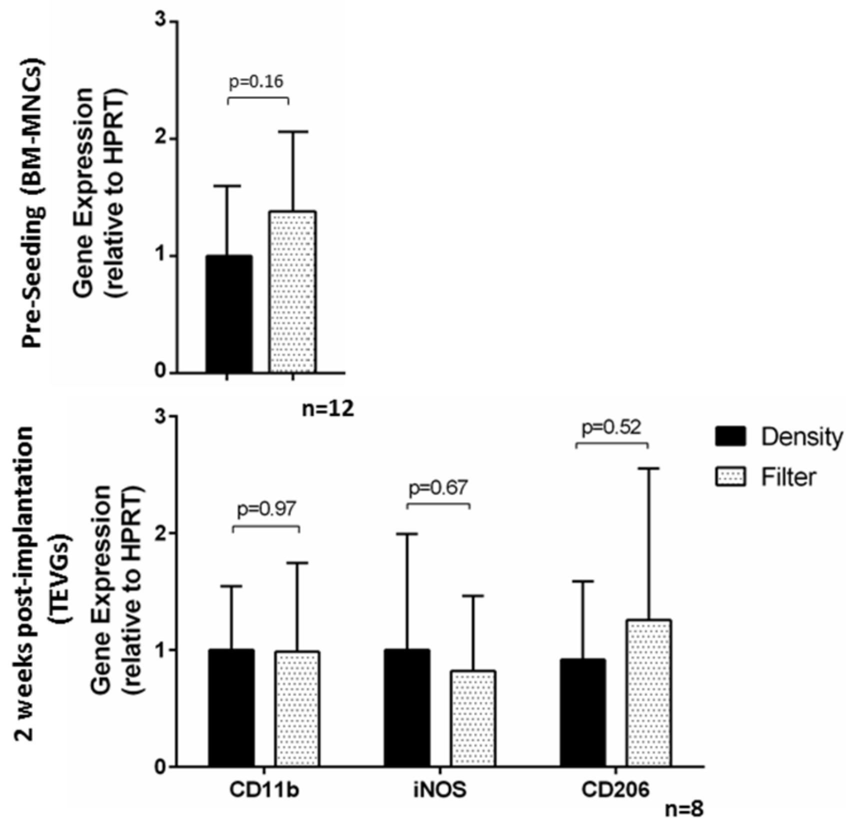

Figure 7. Gene expression of macrophage phenotypes in TEVGs. Gene expression was assessed using the ΔΔ Ct method following real time quantitative reverse transcription polymerase chain reaction (RT-qPCR). Gene expression of the macrophage marker CD11b following isolation (upper panel) and 2 weeks after implantation (lower panel) showed no difference between the filter and density centrifugation methods. In addition, gene expression of the macrophage M1 phenotype marker iNOS and M2 phenotype marker CD206 was not significantly different 2 weeks after implantation. This data was analyzed using the Student's *t* test.

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