Original Article

Linear Shear Conditioning Improves Vascular Graft Retention of Adipose-Derived Stem Cells by Upregulation of the $\alpha_5\beta_1$ Integrin

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Use of adult adipose-derived stem cells (ASCs) as endothelial cell substitutes in vascular tissue engineering is attractive because of their availability. However, when seeded onto decellularized vascular scaffolding and exposed to physiological fluid shear force, ASCs are physically separated from the graft lumen. Herein we have investigated methods of increasing initial ASC attachment using luminal precoats and a novel protocol for the gradual introduction of shear stress to optimize ASC retention. Fibronectin coating of the graft lumen increased ASC attachment by nearly sixfold compared with negative controls. Gradual introduction of near physiological fluid shear stress using a novel bioreactor whereby flow rate was increased every second at a rate of 1.5 dynes/cm^2 per day resulted in complete luminal coverage compared with near complete cell loss following conventional daily abrupt increases. An upregulation of the $\alpha_5\beta_1$ integrin was evinced following exposure to shear stress, which accounts for the observed increase in ASC retention on the graft lumen. These results indicated a novel method for seeding, conditioning, and retaining of adult stem cells on a decellularized vein scaffold within a high-shear stress microenvironment.

Introduction

BYPASS OF OCCLUDED VESSELS continues to be an important and common technique to treat peripheral arterial and coronary artery disease. Autologous vascular tissue remains the gold standard conduit for surgical bypass, providing an effective and versatile arterial substitute.[1] Unfortunately, availability of autologous tissue can be problematic,[2] as up to 40% of patients lack adequate tissue for successful surgery.[3,4] In the absence of autologous tissue, surgeons may rely on alternative conduits such as prosthetic grafts. When compared with autologous tissue, prosthetic grafts have significantly lower patency rates and increased frequency of infection.[3,5,6] Given these issues, a significant need exists to design an improved alternative; vascular tissue engineering seeks to create such an option.

The ability of adult stem cells to differentiate into endothelial cells (ECs) makes them candidates for use in vascular tissue engineering.[7–9] Both peripheral blood endothelial progenitor cells and bone marrow-derived stem cells have been used to seed vascular grafts, which showed encouraging results.[10–13] Unfortunately, bone marrow-derived stem cells are available in limited quantities compared with adipose-derived stem cells (ASCs) and present with a significant risk of donor site morbidity.[14,15] Similarly, endothelial progenitor

cells are also available in limited quantities and are difficult to isolate and culture.[16] Our group[17–21] and others[22–25] have shown that adipose tissue is a source of readily available, autologous stem cells with the potential for use as EC substitutes. We have demonstrated that EC growth supplement (ECGS) and shear stress stimulate ASCs to acquire several EC characteristics, such as expression of CD31 (platelet-EC adhesion molecule), uptake of acetylated low-density lipoproteins, alignment in the direction of fluid flow, and formation of capillary-like structures when plated on Matrigel.[26] These characteristics make ASCs the appropriate candidates for the seeding of tissue-engineered vascular grafts.

One of the challenges of vascular graft seeding is retention of the cells on the luminal surface after placement of the graft into the arterial tree, because in this microenvironment, blood flow and resulting shear stress have resulted in the removal of seeded cells. Several strategies have been employed to achieve retention of seeded ECs onto prosthetic grafts, including use of precoats[27] and shear stress conditioning.[28,29] Precoating with extracellular matrix proteins provides an increased number of ligands for attachment via cell surface integrins. Shear stress conditioning involves the gradual introduction of shear stress within the lumen of a seeded graft until physiological levels are achieved without cell loss. Typical protocols involve stepwise, daily increases in shear

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stress over a period of several days. In our initial attempts to seed decellularized vascular scaffolds with ASCs, we observed near complete cell loss following immediate introduction of physiological shear stress (9 dynes/cm^2), as well as stepwise increases of 3 dynes/cm^2 per day over 3 days to 9 dynes/cm^2. Based on this observation, we hypothesized that a gentler, linear increase in shear stress following seeding would improve attachment such that the stem cells are retained at physiologic levels of shear stress.

This study describes the creation of a tissue-engineered vascular graft composed of adult stem cells (i.e., ASCs) seeded onto a natural vascular tissue scaffold (herein, decellularized human saphenous vein allograft). ASCs isolated from the periumbilical fat from patients undergoing vascular surgical procedures were cultured in EC-differentiating medium and seeded onto decellularized vein allografts. Using an *in vitro* assay, we determine the optimal precoat of the graft for stem cell attachment. Within a vascular graft bioreactor, we then compare two patterns of flow conditioning— a stepwise increase in shear versus a linear increase—and demonstrate superior attachment with the linear pattern. Ultimately, a confluent monolayer of endothelial-like stem cells is established upon the luminal surface of the graft at physiological levels of shear stress. Finally, we present data suggesting that an increase in stem cell integrin expression in response to shear stress is responsible for the observed improved attachment.

Materials and Methods

Stem cell isolation and culture

Adipose tissue was obtained via periumbilical liposuction in patients undergoing elective vascular surgery at the Thomas Jefferson University Hospital. All patients were consented, and donations were conducted under an Institutional Review Board-approved protocol. ASCs were isolated and characterized as previously described,[26] and an adult stem cell population with a CD13$^+$29$^+$90$^+$31$^-$45$^-$ phenotype as determined by flow cytometry was obtained. Isolated ASCs were differentiated by culturing in M199 media (Mediatech, Herndon, VA) containing 10% fetal bovine serum (Gemini BioProducts, West Sacramento, CA), HEPES buffer (1 M; Fisher, Pittsburgh, PA), heparin (Elkins-Sinn, Cherry Hill, NJ), antibiotic/antimycotic solution (Mediatech), and ECGS (6 µg/mL; BD Biosciences, San Jose, CA) for a period of 2 weeks. The ASCs utilized in all experimental procedures were between passages 2 and 6. Care was taken to not allow ASCs to become over confluent. Human umbilical vein ECs (HUVECs) were used as positive EC controls.

In vitro *cell attachment assay*

Ninety-six–well plates (Falcon, BD Labware, Franklin Lakes, NJ) were coated with collagen I (150 µg/mL; BD Biosciences), collagen IV (10 µg/mL; BD Biosciences), fibronectin (18 µg/mL; BD Biosciences), 100% fetal bovine serum, ECGS media, or sterile water (negative control). Following precoating, as per manufacturer's instructions, the solutions were aspirated. ASCs were harvested with trypsin, which was inactivated with serum-containing media, and cells were pelleted via centrifugation (300 g, centrifugal radius = 17.5 cm). ASCs were resuspended in serum-free M199 media, placed

into precoated wells (1×10^5 cells/well), and allowed to attach for 1 h at 37°C. Each well was gently washed with phosphate-buffered saline (PBS), and the adherent cells were fixed with 3.7% paraformaldehyde for 10 min at room temperature. The cells were stained with crystal violet in 20% methanol for 5 min, followed by several PBS washes, after which the dye was eluted from the attached cells with 0.1 M sodium citrate (pH 4.2) overnight. The absorbance of each well was measured at 590 nm. Cell attachment was determined by normalizing the absorbance of each well to that of the negative control (wells precoated with sterile water).

Vascular graft preparation and seeding

Human saphenous veins obtained from a cadaver tissue bank (Regeneration Technologies, Birmingham, AL) were cleaned and decellularized using 0.075% sodium dodecyl sulfate, as previously described and characterized by us.[20] A decellularized vein segment (4 ± 1 cm in length and 0.5 ± 0.1 cm in outer diameter) was mounted within a bioreactor chamber (LumeGen System; Tissue Growth Technologies, Minnetonka, MN). The closed system bioreactor chamber was filled with growth media and contained within an incubator maintained at 37°C and 5% CO$_2$. Vessel diameter and length were acquired using digital calipers and the vein volume and lumen surface area were calculated. The graft was filled with fibronectin (18 µg/mL in dH$_2$O; BD Biosciences) and incubated at 37°C with rotation around the longitudinal axis (5 rpm) for 1 h. Subsequently, ASCs were harvested with trypsin, resuspended in cell culture media, perfused into the graft lumen at a concentration of 2×10^5 cells/cm^2 luminal surface area, and incubated at 37°C with rotation around the longitudinal axis at 5 rpm for 2 h.

Bioreactor design and shear conditioning

A LabVIEW Virtual Instrument (National Instruments, Austin, TX) controlled the speed of a peristaltic pump (Watson Marlow, Wilmington, MA) that drove the flow of media through the lumen of ASC-seeded vascular grafts. The flow rates to achieve desired shear stresses were calculated using the following equation[30,31]:

$$\tau_{wall} = \frac{4\mu v}{R}$$

where τ is shear stress, µ is the viscosity of the media, ν is the average velocity of media flow (mL per min/cross-sectional area), and R is the internal radius of the graft. The smooth, linearly increasing flow pattern was achieved by dividing the maximum flow rate by the number of seconds desired for the length of experimentation (3 dynes/cm^2 per day for 3 days or 1.5 dynes/cm^2 per day for 6 days).

Graft visualization and quantification

Scanning electron microscopy. Vascular graft samples were fixed in 3.7% paraformaldehyde for a minimum of 1 h. The tissue was then dehydrated with an ethanol series (50%, 70%, 80%, 90% 95%, and 100%; 30 min each). Final dehydration was achieved by incubation of the tissue in Freon overnight. The dehydrated samples were gold sputter coated and imaged with a Hitachi TM-1000 Tabletop Electron Microscope (Hitachi High Technologies America Inc., Pleasanton, CA).

Laser confocal microscopy. To quantify cell retention following shear stress application, the grafts were opened longitudinally, stained with Cell Tracker Green, Alexa Fluor 488 phalloidin, and/or propidium iodide (Invitrogen, Carlsbad, CA), as per manufacturer's instructions, and visualized by laser confocal microscopy (488 and 543 nm). For each sample, 10 individual, random $100\times$ images were captured, which represented $>75\%$ total experimental graft area. Using ImagePro software, the total percentage of cell area coverage was determined for each image.

Orbital motion induced shear stress

Shear stress was applied to confluent ASC cultures grown in six-well plates with an orbital shaker (Bellco, Vineland, NJ) as described previously.[32–34] This technique applies a variable degree of shear stress across the monolayer, with a maximal level determined using the following equation:

$$\tau_{max} = \alpha\sqrt{\rho\eta}(2\pi f)^3$$

where α is the radius of orbital rotation, ρ is the density of the culture medium (1.0 g/mL), η is the viscosity of the medium (0.01 poise), and f is the frequency of rotation (rotations/s). Specifically, for our experimental parameters (six-well plates, 2 mL ECGS media), a rotational frequency of 210 rpm results in a shear stress of 10–15 dynes/cm^2 across the majority of the cellular monolayer.

Integrin-mediated cell adhesion assay

ASC integrin expression was quantified using the Alpha/Beta Integrin-Mediated Cell Adhesion Array Combo Kit (Chemicon International–Millipore, Billerica, MA) as per manufacturer's protocol. ASCs were exposed to orbital fluid shear or static conditions for 48 h, harvested using trypsin, plated in the experimental wells at a concentration of 5×10^4 cells/well, and then allowed to attach for 1 h at 37°C. Each well contained a specific mouse monoclonal antibody generated against human alpha and beta integrins/subunits which were immobilized onto a goat anti-mouse antibody-coated microtiter plate. Following incubation, the cells were washed with PBS and stained with crystal violet. The wells were washed and stain was eluted from attached cells, followed by absorbance measurement at 590 nm.

Cell integrin blockade

Prior to seeding within the tissue culture wells, the ASCs were pretreated with a human monoclonal antibody against the $\alpha_5\beta_1$ integrin (MAB 1969; Chemicon International–Millipore, Billerica, MA) at a concentration of 25 µL/mL for 1 h.

Quantitative polymerase chain reaction

Total RNA was isolated from ASCs using RNeasy Mini Spin Columns (Qiagen, Valencia, CA). One microgram of total RNA was reverse transcribed (Reverse Transcription System; Promega, Madison, WI), followed by quantitative polymerase chain reaction (PCR) executed using TaqMan Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA) in a 96-well format (7500 Fast System; Applied Biosystems). The reactions were internally controlled with glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The following TaqMan Gene Expression Assays (Applied Biosystems) were used: integrin alpha 5, Hs00233743_m1; integrin beta 1, Hs00559595_m1; GAPDH, Hs99999905_m1.

Western blot

Experimental cell cultures were harvested with ethylenediaminetetraacetic acid (50 mM; Promega) to preserve integrin integrity. Trypsin was not used in the harvest of protein lysates because it causes physical disruption of integrins. Cell pellets were treated with RIPA buffer containing protease and phosphatase inhibitors (Thermo Scientific, Waltham, MA). Cell debris was removed via centrifugation. Protein concentration was quantified with a bicinchoninic acid (BCA) colorimetric protein assay (Thermo Scientific). Protein was size separated on 8% Tris-Glycine gels (Invitrogen) and transferred to polyvinylidene fluoride membranes (Invitrogen). Blots were blocked in fat-free milk. Primary antibodies were obtained from (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and are as follows: alpha 5, sc-6595 1:1000; beta 1, sc-8978 1:1000; GAPDH, sc-47724 1:10,000. Horse radish peroxidase-conjugated secondary antibodies were used at a concentration of 1:10,000. Blots were developed with a chemiluminescent detection system (Immobilon Western–Millipore, Billerica, MA).

Data analysis

The results are expressed as mean ± standard error of the mean. Comparisons between groups were conducted by one-way analysis of variance; $p < 0.05$ was considered statistically significant. All experiments were repeated $n = 3$.

Results

Optimization of stem cell attachment to the vascular scaffold

The use of decellularized human saphenous vein as a scaffold for vascular tissue engineering provides the benefit of retaining the extracellular matrix and basement membrane integrity, thereby providing natural ligands for ASC attachment.[20] However, preliminary cell retention studies determined that ASCs seeded onto this scaffold, without additional luminal precoating, were completely dislodged by 9 dynes/cm$^2\times$24 h of fluid shear stress (Fig. 1). This result suggested the need for improvement of our graft creation protocol, including both precoating and flow conditioning.

To determine the optimal precoat, we performed an *in vitro* cell attachment assay whereby ASCs were seeded onto plastic tissue culture dishes coated with various proteins associated with vascular tissue extracellular matrix, basement membrane, and cell attachment. Although all of the tested proteins improved ASC attachment compared with negative control, collagen I and fibronectin demonstrated the largest increase in ASC attachment (more than fivefold; Fig. 2).

To arbitrate the optimal incubation time for initial ASC attachment, we quantified attachment to the vascular scaffold as a function of time. At unit gravity, the stem cells begin to attach to the luminal surface in 30 min with maximal attachment at 2 h (Fig. 3A). HUVEC controls demonstrated similar results. Although further static culture over 24 h demonstrated no significant gain or loss in number of ASCs attached to graft

FIG. 1. Poor retention of stem cells when exposed to shear stress without flow conditioning. Laser confocal micrographs (Cell Tracker Green; $\times 10$) of human adipose-derived stem cells (ASCs) seeded onto the lumen of decellularized vein allograft (**A**) immediately after graft creation and (**B**) following exposure to shear stress ($9\,dynes/cm^2 \times 24\,h$) demonstrating complete dislodgement of stem cells.
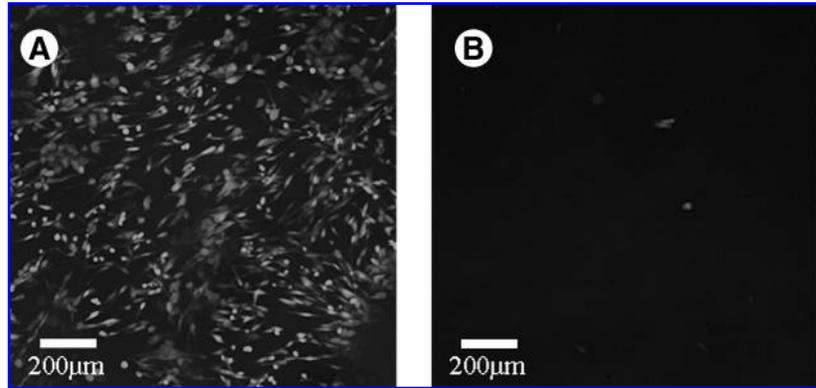
compared with 2 h (initial cell attachment with spherical shape; Fig. 3B), increased incubation time promoted greater cell spreading and a more natural morphology (Fig. 3C).

Following these studies, we concluded that the optimal preparation protocol of our stem cell tissue-engineered vascular grafts would include the following conditions: (1) precoating the decellularized vein lumen with fibronectin, (2) initial seeding of the graft lumen with stem cells at unit gravity for 2 h (including rotation around the longitudinal axis to promote even seeding), and (3) instituting a gentle fluid flow of culture medium ($5\,mL/min$, $0.01\,dynes/cm^2$) through the graft lumen over the next 24 h to allow for optimal cell spreading.

Figure 4 demonstrates the end result of this protocol, imaged after various steps by scanning electron microscopy. Prior to decellularization, fresh saphenous vein samples revealed a completely intact endothelium (Fig. 4A). Following decellularization in sodium dodecyl sulfate, the endothelium has been completely removed revealing the details of the underlying basement membrane (Fig. 4B). After fibronectin coating, luminal seeding (2×10^5 cells/cm$^2 \times 2\,h$), and gentle medium fluid exchange (24 h), ASCs remained attached to the graft surface (Fig. 4C). Interestingly, despite a very low shear stress ($0.01\,dynes/cm^2$) seen during this fluid-exchange period, the stem cells have already begun to align in the direction of flow, suggesting endothelial differentiation and adaptation.

Effect of shear conditioning pattern on stem cell retention

We next evaluated the effect of flow conditioning—the gradual introduction of shear stress—on cell retention. In these studies, two patterns of flow conditioning were examined: (1) a stepwise pattern, whereby shear stress was increased daily by $3\,dynes/cm^2$, for a goal of $9\,dynes/cm^2$ after 3 days; and (2) a gradual application pattern, whereby shear stress was increased linearly from 0 to $9\,dynes/cm^2$ over 3 days. Figure 5 illustrates the results of these studies. Visualization of seeded grafts with confocal microscopy following daily stepwise flow increases shows that both ASCs and ECs begin to detach from the graft lumen after reaching shear forces of $6\,dynes/cm^2$, with complete cell loss at $9\,dynes/cm^2$ (Fig. 5A). Examination of results following linear flow conditioning (Fig. 5B) demonstrated a significant improvement in cell retention for both ASCs and ECs compared with stepwise flow

increases. Quantification of these results by image analysis indicated that the ultimate retention of ASCs on the graft was 45% after linear conditioning compared with 2% after stepwise conditioning (Fig. 5C). In parallel experiments, we observed that linear conditioning improved HUVEC attachment as well (85% vs. 3% attachment, respectively; Fig. 5D).

Role of $\alpha_5\beta_1$ integrin expression on stem cell attachment and retention

Previous work by our group demonstrated that shear stress stimulates ASC differentiation to an EC-like phenotype.[26] Given the critical role of integrins in cell attachment,[35] and to determine a possible mechanism for improved attachment of ASCs with flow conditioning, we examined the role of integrin expression in stem cell attachment and retention.

We first evaluated the effect of shear stress on stem cell attachment within an *in vitro* cell attachment assay model. Differentiated ASCs were either cultured under static con-
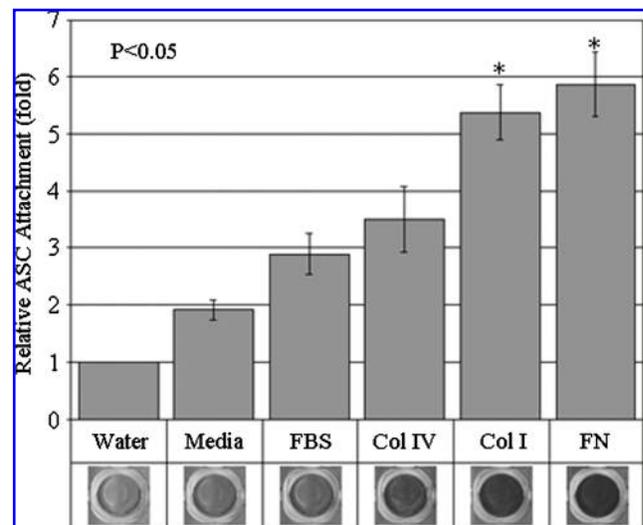

FIG. 2. Effect of precoat on stem cell attachment. ASCs were incubated for 1 h on surfaces coated with dH_2O, endothelial cell growth supplement media alone, 100% fetal bovine serum (FBS), collagen IV (Col IV), collagen I (Col I), and fibronectin (FN). Crystal violet uptake of attached ASCs revealed optimal attachment of stem cells to collagen I and fibronectin. * denotes statistical significance vs. control; $p < 0.05$.
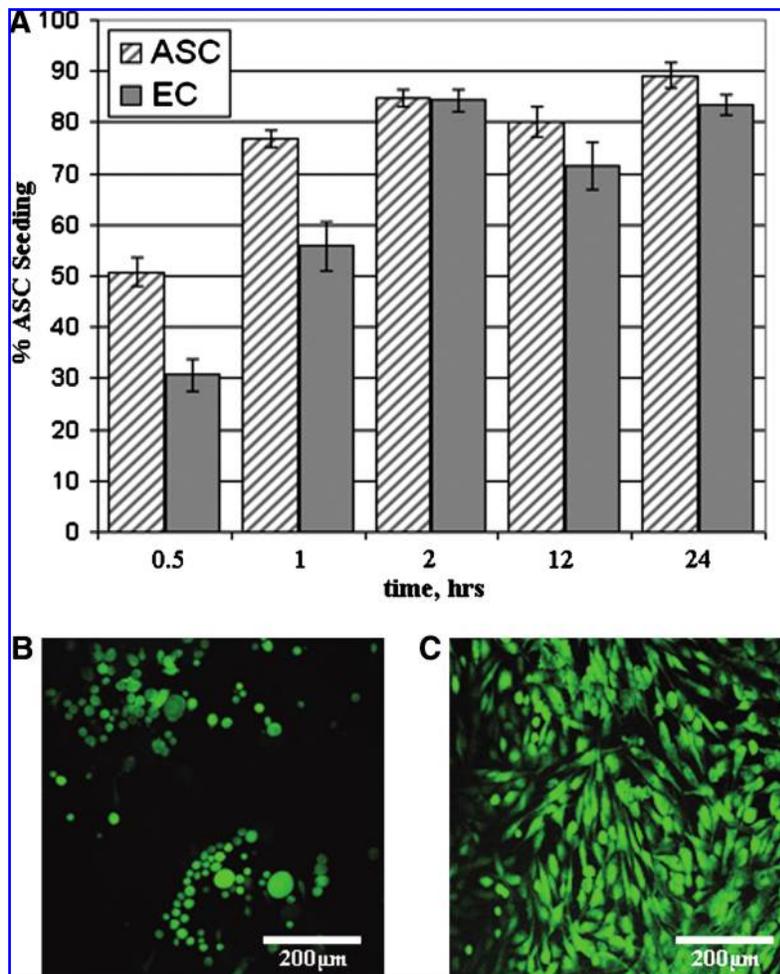
FIG. 3. Initial cell attachment over time. ASC attachment to decellularized vein allograft quantified over time and compared with endothelial cell (EC) attachment. (**A**) Fifty percent of ASCs attach as early as 0.5 h, with maximal attachment observed at 2 h. Further attachment was not seen with initial seeding times as long as 24 h. (**B**) Two hours after initial seeding, ASCs are firmly attached, but have a spherical shape (Cell Tracker Green). (**C**) Twenty-four hours after seeding, the ASCs spread out on the graft and assume a more natural morphology (Cell Tracker Green).

ditions or exposed to shear force using the orbital shaker model (10–15 dyne/cm^2 for 48 h). The cells were then plated onto tissue culture plastic precoated with fibronectin. Compared with statically cultured stem cells, sheared stem cells demonstrated 1.5-fold greater attachment to fibronectin (Fig. 6A, left two bars).

Using an integrin-mediated cell adhesion assay, the expression of integrins α_1, α_2, α_3, α_4, α_5, α_V, $\alpha_V\beta_3$, β_1, β_2, β_3, β_4, β_6, $\alpha_V\beta_5$, and $\alpha_5\beta_1$ were evaluated for static- and shear-cultured ASCs. Most notably, shear stress increased ASC attachment to α_5, β_1, and $\alpha_5\beta_1$ protein antibody-coated wells than statically cultured cells (Fig. 6B). These specific integrins commanded preferential attachment of the sheared ASCs over any other integrin examined.

Examination of stem cell genotype using quantitative PCR after exposure to *in vitro* orbital shear stress revealed an
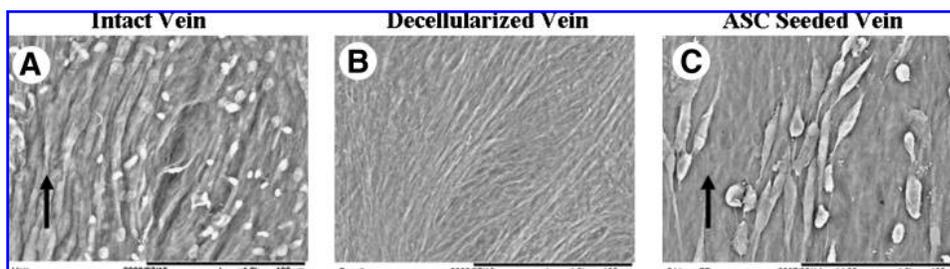

FIG. 4. Replacement of allograft ECs with autologous, differentiated stem cells. Scanning electron micrographs (×1000) of the tissue-engineered stem cell graft during several stages of creation. (**A**) Intact human saphenous vein allograft is completely covered by ECs aligned in the direction of fluid flow (arrow). Red blood cells remain attached to the surface. (**B**) Following sodium dodecyl sulfate decellularization, the ECs have been removed, revealing underlying vascular basement membrane. This surface is representative of the vascular scaffold used in these studies. (**C**) Human ASCs seeded upon the vascular scaffold and cultured under minimal fluid flow for 24 h within the bioreactor. The remaining cells attached and aligned in the direction of fluid flow (arrow).

FIG. 5. Positive effect of linear flow conditioning on cell retention. Laser confocal micrographs (Cell Tracker Green; ×10) of tissue-engineered grafts created with either ASC or EC controls and conditioned to physiological fluid shear stress. (**A**) Initial attempts to increase cell retention under flow with daily stepwise increases of 3 dynes/cm² resulted in significant cell loss at 6 dynes/cm² and near complete loss at 9 dynes/cm² for both ASC and EC controls. (**B**) Gradually increasing fluid shear at a linear rate of 3 dynes/cm² resulted in improved ASC and EC retention at 6 and 9 dynes/cm². (**C**) Quantification of ASC-seeded grafts conditioned with both stepwise and linear application of shear reveals a statistically significant difference in cell retention. Retention was improved to 46% with linear application versus 2% with stepwise application. (**D**) Quantification of EC-seeded grafts demonstrated magnified results. Retention was improved to 85% with linear application versus 3% with stepwise application. * denotes statistical significance vs. control; $p < 0.05$. Color images available online at www.liebertonline.com/ten.
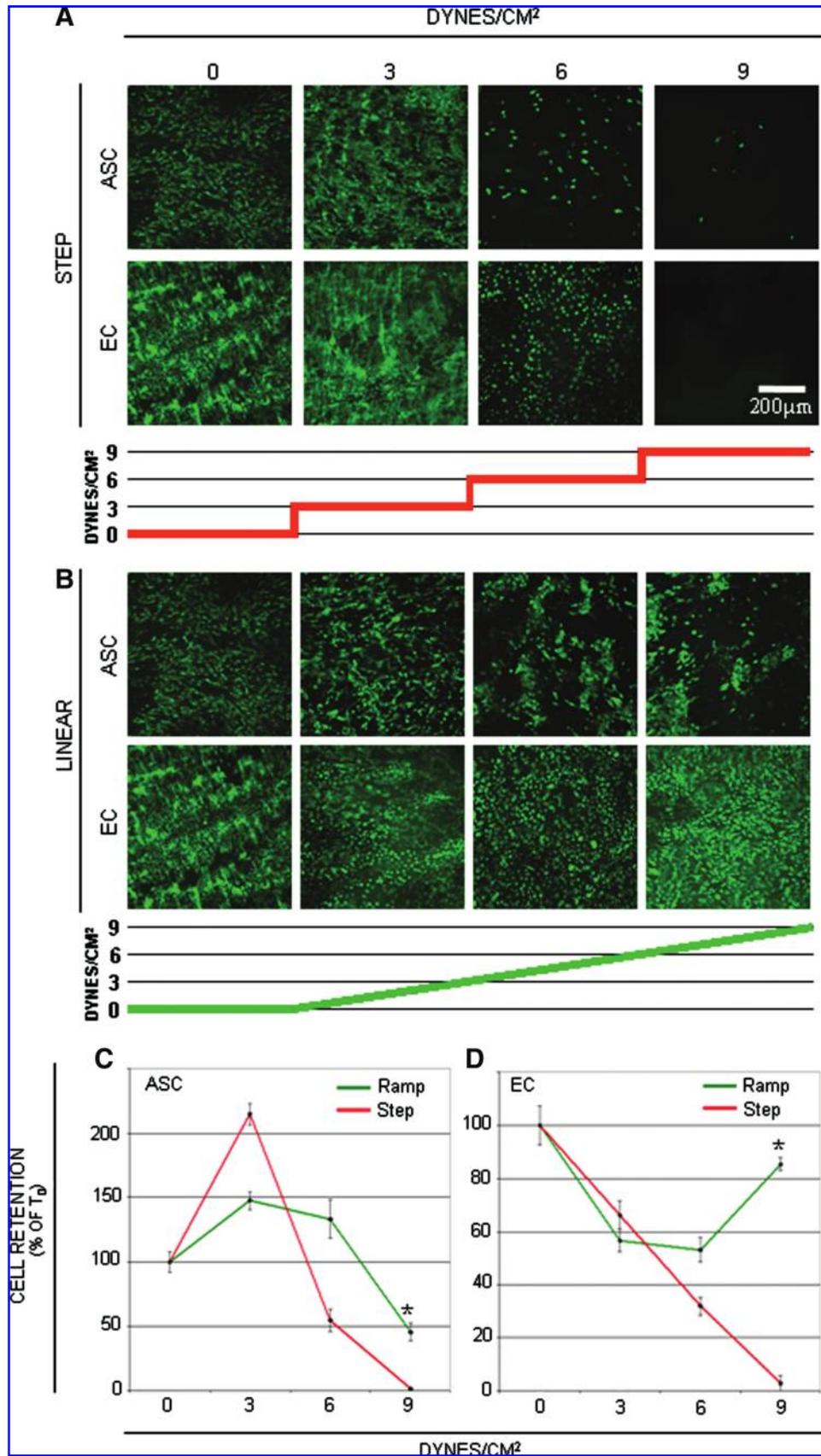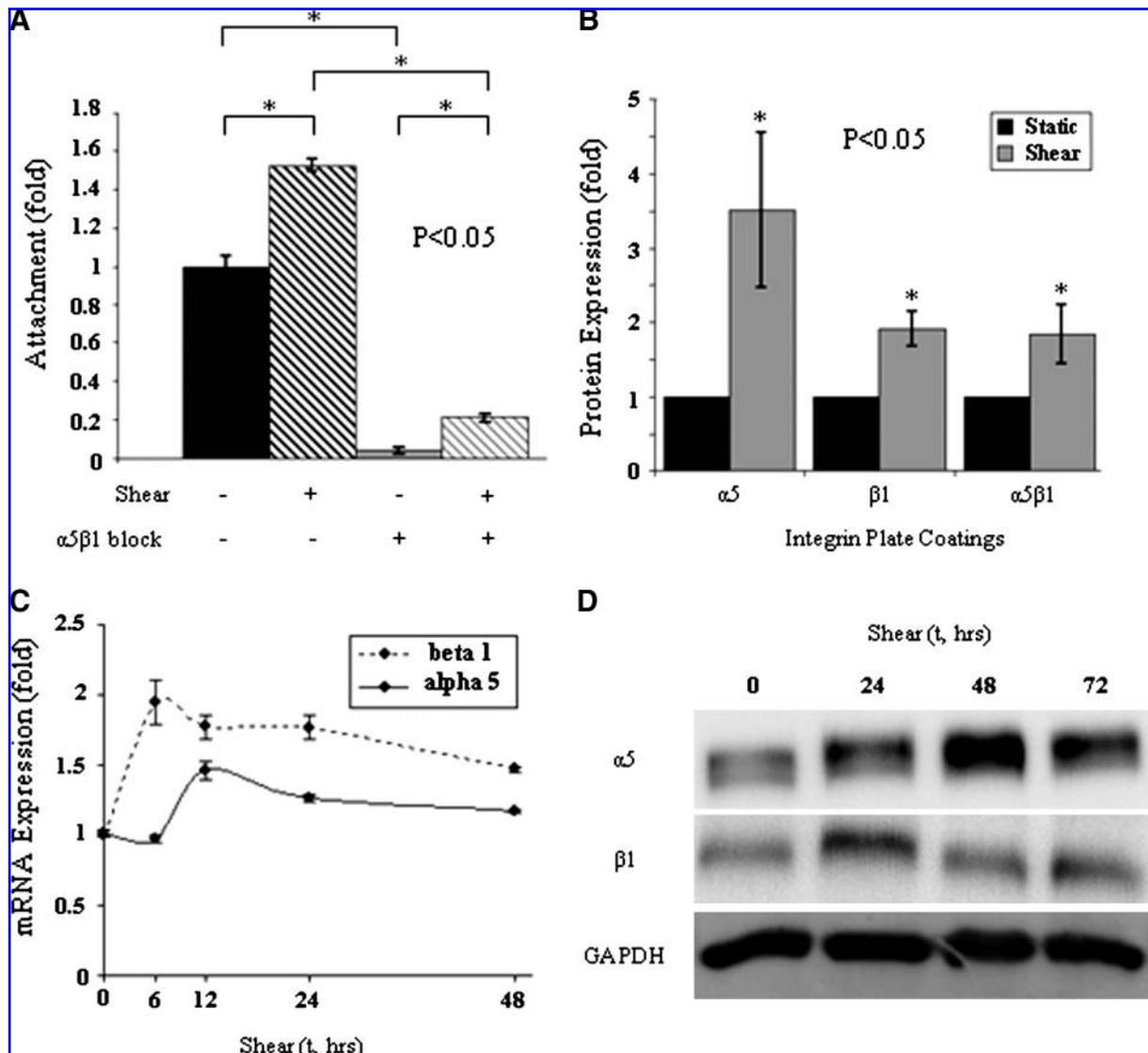
FIG. 6. Analysis of integrin $\alpha_5\beta_1$ expression in ASCs under static and shear conditions. (**A**) Orbital shear stress increases ASC attachment to fibronectin-coated surfaces by 1.5-fold compared to static culture conditions. Attachment of static cultures is reduced by over 90% by $\alpha_5\beta_1$ blockade. Shear cultures demonstrated an 80% reduction in attachment following blockade. (**B**) Integrin-mediated cell adhesion assay. Following determination that ASCs preferentially expressed the α_5 and β_1 monomer units and $\alpha_5\beta_1$ dimer, shear stress was found to significantly increase their expression and resulted in upregulated attachment of the stem cells to surfaces coated with monoclonal antibodies against these proteins. (**C**) Quantitative polymerase chain reaction demonstrated upregulation of α_5 and β_1 mRNA expression with maximal expression at 12 and 6 h, respectively. (**D**) Western blot analysis confirmed upregulation of α_5 and β_1 protein subunits. Taken together, these results implicate the $\alpha_5\beta_1$ integrin as key in the attachment of ASCs to a fibronectin precoated graft and demonstrate a need for acclimation to a changing shear environment due to their temporal upregulation following stress exposure. * denotes statistical significance $p < 0.05$.

upregulation in both α_5 and β_1 integrin mRNA levels compared with statically cultured cells (Fig. 6C). This effect appeared to be time dependant with maximal β_1 and α_5 message level observed at 6 and 12 h, respectively, after exposure to shear stress. Western blot analysis also demonstrated an increase in both α_5 (maximum expression at 48 h) and β_1 (maximum expression at 24 h) protein levels in response to shear stress (Fig. 6D). Additionally, blockade of the $\alpha_5\beta_1$ integrin significantly—and nearly completely—eliminated attachment of both sheared and statically cultured ASCs to fibronectin-coated plates (Fig. 6A, right two bars).

Optimized graft creation

Following the observation that ASCs required 24–48 h to significantly increase $\alpha_5\beta_1$ integrin expression, we modified the flow conditioning protocol to provide extended time for ASCs to acclimate to their changing microenvironment within the bioreactor. The slope of shear stress application was reduced from 3 to 1.5 dynes/cm^2 per day following 24 h of minimal shear (0.01 dynes/cm^2). This new flow profile required a total of 7 days to reach physiological shear levels (9 dynes/cm^2).

Figure 7 illustrates the results of this optimized protocol. A complete monolayer of ASCs was observed over the entire luminal surface at the end of this 7-day conditioning period (Fig. 7A). Figure 7B presents the gross view of the graft created, which is visualized in Figure 7A for comparison. When observed under high magnification, the ASCs were aligned in the direction of fluid flow and actin stress fibers aligned in the direction of flow were also prominent (Fig. 7C). Histological cross sections stained for nucleic acids demonstrated the continuous luminal coverage of the decellularized scaffold with ASCs (Fig. 8). Migration of ASCs throughout vascular tissue layers was not observed over the period of graft creation.

Discussion

We report the successful creation of a tissue-engineered vascular bypass graft utilizing adult ASCs seeded onto the lumen of a decellularized saphenous vein scaffold. Our results introduce a novel method of linear shear conditioning, whereby adult stem cells acclimate to a microenvironment subjected to high levels of mechanical stress yielding optimal stem cell retention on a natural scaffold. Preliminary data revealed that upon abrupt introduction of biomimetic fluid shear stress, ASCs were completely removed from the lumen of a decellularized saphenous vein scaffold. Two improvements were implemented within our graft creation protocol to enhance initial cell adherence and optimize cell retention following exposure to fluid shear stresses as would be found *in vivo*. First, a luminal precoat of fibronectin was demonstrated to increase ASC attachment fivefold. Second, we demonstrated that allowing ASCs to adapt to a gradual increase in shear stress promoted cell retention. To show this, we compared two distinct methods of shear conditioning: (1) daily, stepwise increases and (2) a smooth, linear application

of shear stress. The results indicated that our novel method of gradually increasing the shear optimizes ASC retention, yielding a complete endothelial-like monolayer. The mechanism of this improvement is shown to be mediated, at least in part, by an upregulation in the $\alpha_5\beta_1$ integrin in response to mechanotransduction of fluid-induced shear stress.

In vascular tissue engineering, cell attachment to and retention on an appropriate scaffold is particularly important because of the high fluid shear forces applied to the graft lumen upon implantation within the arterial tree. Synthetic grafts in use today made of materials such as Dacron and expanded polytetrafluoroethylene resist cellular attachment and retention.[36] This limits the use of synthetic materials in tissue engineering applications and encourages the use of natural scaffolds. The work of L'Heureux *et al.* exemplifies the feasibility of the creation and implementation of natural, *de novo* scaffolding; however, they reported a creation time of up to 20 weeks.[37,38] Fortunately, ASCs differentiated toward the endothelial phenotype attach readily to the preserved basement membrane of decellularized vein allograft precoated with fibronectin. This scaffold can be created in 2 days as long as cadaverous donor tissue is available. The addition of a fibronectin precoat increased initial ASC attachment fivefold compared with noncoated surfaces and was determined to serve as the optimal protein for this purpose. Our studies demonstrated optimal ASC attachment to this prepared scaffold after 2 h of incubation, with further cell spreading over 24 h. These results are in agreement with several laboratories that have attempted to overcome the limitations of cell seeding onto vascular grafts using a variety of techniques, including precoating with extracellular matrix proteins such as fibronectin.[39-41]

The microenvironment of an EC in the circulation is under constant mechanical stress, including both fluid-induced cyclical shear stress as a product of the resistance encountered

FIG. 7. Optimized creation of tissue-engineered stem cell graft. Gradual, linear flow conditioning of the stem cell graft (1.5 dynes/cm² per day) resulted in complete cellular coverage at near physiological shear levels (9 dynes/cm²). (**A**) Confocal micrograph (alexa fluor 488 phalloidin, actin stain; ×4) of the graft lumen. (**B**) Gross examination of the graft imaged in (**A**). (**C**) Confocal micrograph (alexa fluor 488 phalloidin, propidium iodide nuclear stain; ×20) revealing alignment of actin stress fibers and focal adhesions. Arrows indicate direction of fluid flow. Arrowheads denote valves. Color images available online at www.liebertonline.com/ten.
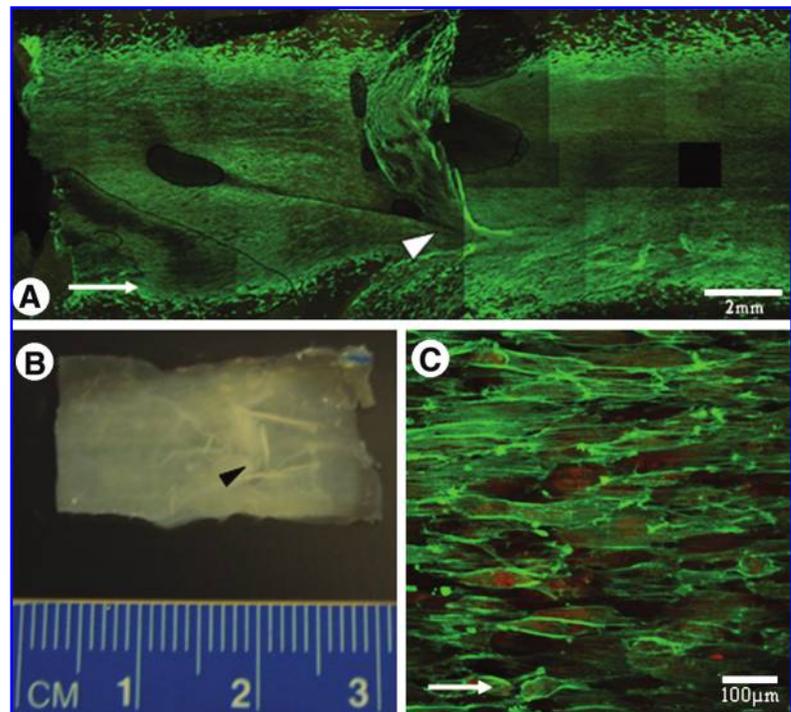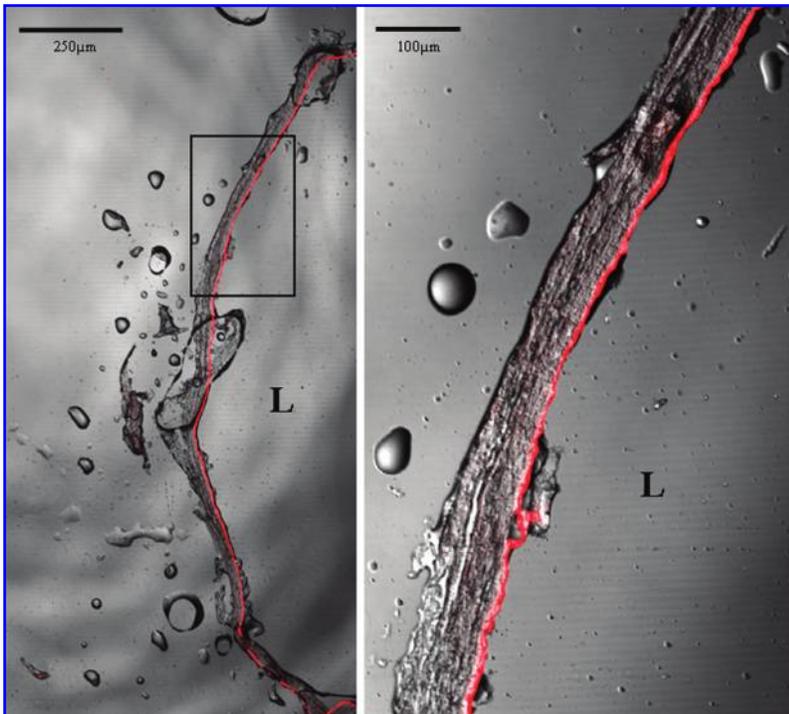
FIG. 8. Neo-intima of stem cell graft is confined to basement membrane. Fluorescence micrograph [propidium iodide; (left)×4, (right) inset×10] of the cross-sectioned stem cell graft demonstrates complete luminal coverage of ASCs on decellularized vascular scaffold. ASCs do not migrate into deeper vascular layers. L, lumen. Fluorescence represents cellular nucleic acid. Color images available online at www.liebertonline.com/ten.

as blood flows through the vessels and longitudinal strain resulting from vessel dilation and relaxation. Cell retention is an even more daunting task for cells used in vascular tissue engineering applications, as the cells will be exposed to *de novo* shear stress. The application of shear stress to ECs has been well documented to increase the cell–matrix binding via integrin mechanotransduction of signals *in vitro*.[42] However, it has been shown repeatedly that immediate exposure of grafts to physiological levels of shear stress results in complete loss of cellular luminal coverage.[27–28,43,44] During the creation of a small diameter vascular graft lined with HUVECs, Inoguchi *et al.*[28] observed a complete loss of luminal cellular coverage following immediate exposure to 8.7 and 19.6 dynes/cm^2 of fluid shear stress. In response to this issue, they conditioned their cells to low levels of shear stress (3.2 dynes/cm^2) for 24 h prior to exposure to the higher shear levels. This stepwise application of shear stress resulted in decreased cell loss. These results were also obtained previously by Dardik *et al.*[43] and Baguneid *et al.*[27] Like these authors, our initial results confirmed a complete loss of seeded cells upon immediate introduction of high shear stress; however, stepwise increases in shear did not condition ASCs for retention on our specific scaffold.

To circumvent this issue, we hypothesized that a gradual application of shear stress would be required for the retention of ASCs on our graft. To advance the technique of shear conditioning, abrupt changes in stress due to stepwise increases in fluid flow were replaced with a gradual, linear increase in shear stress generated by a computer-controlled bioreactor system. This system allows the ASCs to gradually acclimate to their changing environment with the goal of withstanding appropriate levels of shear stress. Linear shear increase of 3 dynes/cm^2 per day over 3 days (end shear 9 dynes/cm^2) resulted in increase of ASC retention to 48% compared with 2% with stepwise application. These results were similar to that of HUVEC-seeded control grafts with an increase to 85% luminal coverage compared with 3%. These results, although improved, were not optimal as the resultant graft lacked a confluent luminal monolayer of stem cells. To optimize cell retention, the slope of shear introduction was reduced to 1.5 dynes/cm^2 per day to allow additional time for ASCs to acclimate to their changing environment. The resulting grafts demonstrated excellent ASC retention on the entire luminal surface, in addition to the alignment of cells in the direction of fluid flow and the formation of actin stress fibers in the same direction. The resultant graft now appeared appropriate for arterial implantation.

The mechanism of how the gradual increase in shear stress results in improved ASC attachment and retention was evaluated. Following exposure to physiological shear forces, a large upregulation was observed for the $\alpha_5\beta_1$ integrin, which when blocked by inactivating antibodies nearly eliminated the attachment of ASCs to fibronectin-coated plates. Evaluation of α_5 and β_1 integrin subunit mRNA and protein levels suggests a temporal aspect to their upregulation. These data confirmed that the gradual introduction of shear force promotes ASCs to adapt to their microenvironment through upregulation of integrins specific to the fibronectin-coated vascular scaffold. Following the binding of an integrin to its matrix ligand, integrins cluster to form focal adhesion complexes that are responsible for strong interactions between cells and their surrounding matrix.[35] Integrins themselves have been implicated in stress sensing and mechanotransduction of extracellular mechanical cues which influence cytoskeletal dynamics, cell motility, cell proliferation, gene expression, cell differentiation, and cell survival.[45]

Research into the attachment and retention of stem cells onto scaffolds is often overlooked when their differentiation is of primary focus. Our laboratory and others have demonstrated an important role of shear stress in the differentiation of stem cells toward an endothelial phenotype. Further, we have reported an easily implemented and feasible

methodology for the construction of a tissue-engineered vascular graft within 3 weeks of time from adipose tissue harvest. The current results advance the role for shear stress from differentiation of ASCs toward an endothelial-like phenotype to include the need of gradual, linearly increasing shear conditioning to allow integrin expression and function for retention within the vascular niche.

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Disclosure Statement

No competing financial interests exist.

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