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Eph-B4 Activation Reduces Neointimal Hyperplasia In Human Saphenous Vein

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EPH-B4 ACTIVATION REDUCES NEOINTIMAL HYPERPLASIA IN HUMAN SAPHENOUS VEIN

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Despite the rise in percutaneous interventions, bypass surgery is an essential therapy for patients with advanced coronary and peripheral artery disease. Nearly half of the saphenous vein grafts used in these operations are eventually complicated by pathologic remodeling known as neointimal hyperplasia (NIH). Recent work in animal models has shown that the development of NIH can be reduced by stimulation of the receptor tryrosine kinase Eph-B4 by systemic injections of its ligand ephrin-b2. The purpose of this study was to determine whether the reduction of intimal hyperplasia observed with Eph-B4 stimulation in animal models translated into human tissues. Specifically we sought to determine whether the development of intimal hyperplasia in human saphenous vein (HSV) tissue could be reduced with ephrin-b2/fc treatments. We also investigated whether Eph-B4 stimulation in human endothelial cells caused similar downstream signaling changes observed in animal cells. Finally we wanted to assess endothelial Eph-B4 stimulation was achievable with adventitial delivery of ephrin-B2/fc. Excess saphenous vein from patients undergoing coronary artery bypass surgery was taken from the OR and put in organ culture for 14 days with or without treatment with ephrin-b2/fc. At the end of 14 days histologic measurements of the intima:media (I:M) were used to track the development of (NIH). The mean baseline I:M ratio was 0.456 ± 0.097 (n=19). Over 14 days in organ culture this ratio increased to 0.726 ± 0.142 (n=19) in untreated veins and 0.630 ± 0.132 (n=19) in treated veins which was significantly different (p=.017). Using quantitative reverse transcription

polymerase chain reaction (qPCR) analysis gene expression changes in organ culture were measured. Eph-B4 and ephrin-b2 decreased while osteopontin expression increased in both treated and untreated groups. Using a human endothelial cell line, we observed increases in phosphorylated AKT and caveolin-1 with ephrin-b2/fc treatment. Finally using an arterial flow model bio-reactor we demonstrated that endothelial eph-B4 phosphorylation was achievable with adventitial application of ephrin-B2/fc in a pluronic hydrogel after 24 hours in arterial flow. In conclusion we show that Eph-B4 stimulation reduces the NIH development *in vitro* in HSV organ culture. Eph-B4 stimulation activates similar signaling cascades in human endothelial cells as in mice. Finally that local delivery of eprhin-b2/fc is possible through a pluronic hydrogel applied to the adventitia of a vein. Combined these results continue to point to Eph-B4 stimulation as a promising target for a therapy to reduce NIH in bypass recipients, however, longer term studies in large animals are necessary.

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Introduction:

Bypass Surgery and Graft Failure

In 1906 Dr. Jose Goyanes of Spain performed the first successful vein graft in humans. He used a section of popliteal vein as an interposition graft in the popliteal artery following a syphilitic aneurysm resection (1, 2). Since then surgeons have employed autogenous vein to bypass arterial circulation for over 100 years. Dr. René Favaloro of Argentina was the first surgeon to use a vein graft to bypass a coronary artery in 1967 (3). Today cardiovascular disease is the leading cause of death in the United States (4). Peripheral artery disease (PAD) is also a major source of morbidity and mortality with an estimated prevalence of 9-12 million Americans which is sure to grow with the increasing age and prevalence of diabetes in our patient population (5). Despite the meteoric increase in endovascular interventions, as much as 300% over the last decade in some academic training programs, bypass surgery remains an important therapy in treating advanced coronary and peripheral artery disease (6, 7). Approximately 250,000 coronary artery bypass graft (CABG) procedures and 80,000 lower extremity bypass surgeries are performed per year in the United States (8).

Within bypass surgery, saphenous vein remains the most commonly used and durable conduit for arterial reconstruction. Despite this a significant portion of saphenous vein grafts are eventually complicated by excessive venous remodeling resulting in graft thrombosis, failure and the associated patient morbidity and mortality. Approximately 20% of coronary artery bypass are no longer patent at 1 year and this number increases to 40% at 5 years (9). In the peripheral arterial

system patency of the graft can vary depending on the location of the distal anastomoses as well as the indication for the bypass such as claudication versus critical limb ischemia. In a recent large study 1 year overall patency rates for bypasses for critical limb ischemia was 61%(10). 5 year patency of grafts employed for claudication had variable 5 year patency rates ranging from 48-81% depending on the anatomic level of distal anastomoses (11, 12).

It has long been known that autogenous vein graft patency exceeds that of artificial conduits such as polytetrafluoroethylene (PTFE)(13). However we have also observed that that internal mammary artery grafts employed in CABGs exhibit better long term patency than saphenous vein grafts. (14) This variation in patency rates both in respect to location, host status, and material underline the dynamic nature of the biologic processes that vein grafts undergo in the arterial system. Thus, understanding the molecular mechanisms involved in long term graft patency as well as possible pathways for improving patency rates will remain an important goal of research.

Vein Graft Adaptation and Neointimal Hyperplasia

Under normal physiologic conditions the vein is a thin walled, highly compliant, vessel. The vein undergoes lower pressures, shear stress, oxygenation, and flow than the artery. Both the arterial and venous vessel wall are composed of three layers: the intima, media, and adventitia. The media of the vein is composed of a thin layer of vascular smooth muscle whereas the arteries possess a much thicker media and adventitia. Veins also contain less elastic tissue than arteries. One final

important difference between the vein and the artery is that the vein has intraluminal valves to prevent the reverse flow of blood. Overall, the thinner vessel wall of the vein wall allows for a more compliant vessel that adapts to physiologic fluctuations in volume load and pressure while returning blood to the heart. However, this thin walled venous architecture is disrupted when the vein is taken from the low flow, low pressure, low shear stress venous circulation and place into the high flow, high pressure, high shear stress arterial circulation during bypass surgery.

Since the 1950s investigators have noted both the gross and histologic changes that veins undergo following anastomosis into the arterial circulation. Barker et al., first referred to these changes as 'arterialization' and since then numerous groups have detailed the changes in the vessel wall of the vein graft (15, 16). These changes include an increase in overall diameter accompanied by a thickening in width of all three layers of the vessel wall: intima, media, and adventitia (Figure 1). The intima undergoes the most drastic changes. In the normal vein the intima is a single layer of squamous epithelium. Following implantation into the arterial system, the intima of the vein graft is invaded by smooth muscle cells which proliferate and deposit extracellular matrix (17). This thickening of the intima has been described histologically as 'fibrous proliferation', 'fibrocellular thickening' or grossly as a 'firm intramural nodule of white, fibrous tissue…covered by smooth tan endothelium'(18-20). We now term this pathologic thickening as 'neointimal hyperplasia'.

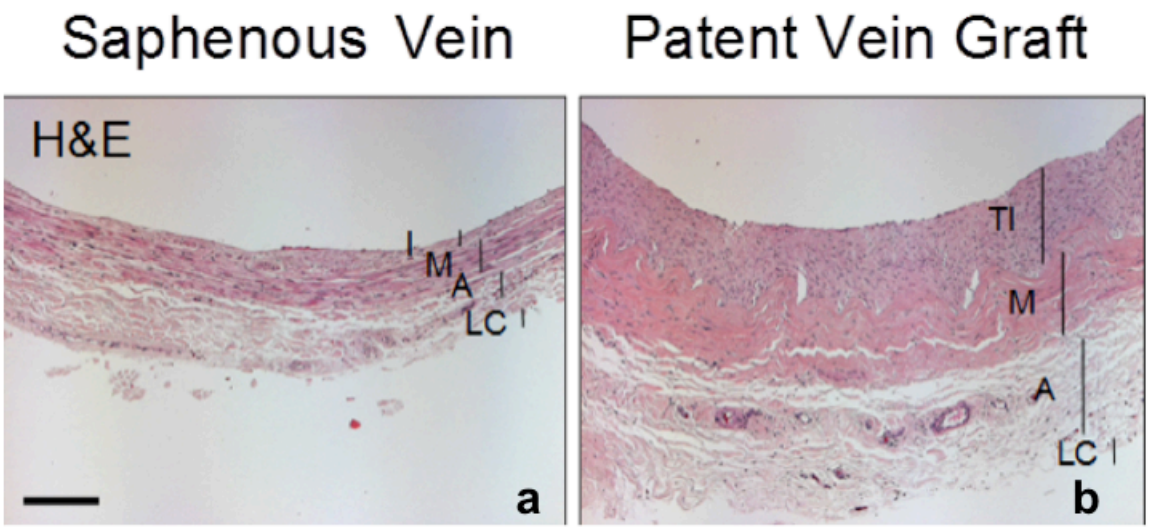

Figure 1. H&E histology of normal saphenous vein (a) and patent vein graft (b). Image taken from Kudo et al., 2007(21)

The complex set of structural and molecular changes that the vein graft undergoes when placed in arterial circulation are a necessary adaption to the demands of the arterial environment. This adaptive process is referred to as vein graft adaptation. Neointimal hyperplasia refers to the excessive remodeling which leads to stenosis and compromise of graft patency. In a 2005 review Mitra et al offered a useful chronologic framework to view both the stimuli and molecular mechanisms for the changes to the vessel wall (17). These steps are: initial endothelial cell damage, platelet activation, leukocyte recruitment and subsequent inflammation, activation of the coagulation cascade, and smooth muscle cell migration and proliferation.

Harvesting of the saphenous vein in bypass surgery is a traumatic event for the vein that directly affects both the endothelium and adventitia of the vein. While some surgeons employ an *in situ* technique which allows the majority of the vein graft to remain in its anatomic position and avoids disruption and stripping of the

adventitia, most peripheral bypasses and all cardiac bypass require that the vein be removed from the surrounding tissue and the side branches ligated prior to anastomosis into the arterial circulation. During harvest, the vein not only undergoes direct surgical trauma but is also separated from the vasa vasorum leading to hypoxia and release of inflammatory cytokines (22). While the saphenous venous endothelium usually experiences shear stresses around 0-4 dynes/cm^2, this number immediately rises to 25-30 dynes/cm^2 in the arterial circulation which generates reactive oxygen species (23). Trauma, hypoxia, and the hydrostatic forces combine to disrupt the endothelium which reduces its ability to release nitric oxide and regulate intimal growth (24).

Disrupted endothelium leads to platelet adhesion, accumulation, and activation which in turns causes the release of multiple bioactive substances including platelet derived growth factor (PDGF) and thromboxane A2 which are smooth muscle cell mitogens (25). Other factors are released including TGF-β, Il-1, Il-6, and Il-8 (26). Platelet activation initiates leukocyte adherence, recruitment, and activation with subsequent chronic inflammation over the following days to week. Chronic inflammation has long been associated with restenosis. Thus many therapies aimed at inhibiting neointimal hyperplasia have focused on reducing inflammation such as blockade of the MCP-1 receptor, transfection of the anti-inflammatory vaccine virus protein 35K, flavanoid treatment, and perivascular treatment with rapamycin which have all shown to reduce neointimal hyperplasia in animal models as reviewed in Muto et al (26). Disruption of the endothelium also leads to exposure of tissue factor which in turns activates the coagulation cascade

leading to both thrombin production and further platelet release of PDGF which are both known to lead to smooth muscle cell proliferation (17).

The biology behind smooth muscle cell migration and proliferation during the first month of vein graft adaptation is the most studied component of neointimal hyperplasia (17, 26, 27). Quiescent 'contractile' smooth muscle cells in the media react to the above mentioned multiple stimuli of vein grafting and migrate into the intima become motile 'synthetic' smooth muscle cells. Smooth muscle cell proliferation is promoted by the growth factors PDGF, insulin growth factor-1 (IGF-1), fibroblast growth factor (FGF), TGF-β, thrombin, and the cytokines IL-1, and IL-6 which are derived from the endothelium, immune cells, platelets, and smooth muscle cells themselves (28). Both the MAPK and AKT intracellular pathways have been implicated in promoting smooth muscle cell proliferation. ERK 1/2 MAPK antagonism led to a decrease in medial cell proliferation and intimal hyperplasia in a canine model of vein grafting (29). Similarly inhibition of the PI3K-Akt/PKB pathway via PTEN overexpression reduced intimal hyperplasia and smooth muscle cell proliferation in a separate canine experiment (30). Both of these pathways are known to contribute to cellular mitogenic, proliferation, and survival pathways and thus their inhibition leading to a reduction in intimal hyperplasia further implicates smooth muscle cell proliferation as a central event in neointima formation.

The importance of extracellular matrix degradation and deposition during vein graft adaptation has been increasingly recognized in recent years. Early in the development of neointimal hyperplasia, extracellular matrix deposition is required for smooth muscle migration and matrix metalloproteinases (MMPs) inhibition has

been shown to reduce neointima formation (31). Similarly overexpression of the tissue inhibitor of metalloproteinase-3 (TIMP-3) reduced neointima formation as well. (32) Extracellular matrix deposition is equally important to vein graft adaptation. Up to 80% of the neointima may be composed of matrix. This combined with continued upregulation of TGF-β for 1-6 months following vein graft implantation demonstrate the importance of matrix deposition in both the early and long term vein graft adaptation (33).

The PREVENT Trials

Historically smooth muscle cell proliferation have occupied a central role in the study of venous neointimal hyperplasia. The focus on smooth muscle cell proliferation likely derives from their dominant cellular contribution to the neointima lesion as well as the perceived similarities between the more widely studied arterial response to angioplasty injury and venous neointima formation. Thus the first molecular therapy directed at inhibiting vein graft neointimal hyperplasia used the E2F transcription factor decoy, edifoligide. Edifoligide was a double-stranded deoxynucleotides that served as a competitive inhibitor for E2F family of transcription factors which are necessary for smooth muscle cells to progress through the cell cycle and proliferate (34). The drug was administered by a one time delivery of pressurized deoxynucleotide containing solution on the back table of the operating room following harvest of the saphenous vein but prior to implantation as a vein graft (35). Unfortunately large randomized controlled trials

of treatment with the E2f decoy in both peripheral and cardiac bypass grafts did not improve long term patency (35, 36).

Eph-B4 and Venous Identity: A Novel Target for Neointima Inhibition

While there are numerous possible reasons that the PREVENT trials did not succeed, the Dardik lab hypothesized that a novel target derived not from smooth muscle biology but venous biology may yield a promising target in inhibiting neointimal hyperplasia. In recent years, the critical role of Eph (erythropoietin producing hepatocellular carcinoma) family of tyrosine kinases and their ligands (Ephrin) in the process of venous arterial identity during development has emerged (37, 38). Operating via Sonic Hedgehog, VEGF, and Delta-Notch signaling cascade, ephrin-B2 expression is an embryonic determinant of arterial fate whereas this cascade is blocked in by the COUP-TFII transcription factor in venous endothelium leading to Eph-B4 expression (39, 40). While both Eph-B4 and ephrin-B2 are expressed throughout adulthood, it was uncertain whether they remained simply as markers of vessel identity or persisted as plastic determinants of vessel fate especially during venous adaptation.

Using a rat model of vein grafting, the Dardik lab demonstrated that Eph-B4 expression was lost during vein graft adaptation corresponding to a loss of venous identity without a concomitant rise in ephrin-b2 (21). These experiments also identified that the majority of Eph-B4 expression originated in the endothelium although there was some Eph-B4 expression in smooth muscle cell layers. The loss

of Eph-B4 expression was also seen in patent saphenous vein grafts excised from transplanted human hearts (21).

To test whether Eph-B4 signaling could be manipulated during vein graft adaptation vein grafted mice were injected with multiple, systemic injections of ephrin-B2, intimal thickness of the vein graft could be reduced (41). Further demonstrating Eph-B4's role vein graft adaptation, Eph-B4 heterozygous knockout vein grafts demonstrated increased intimal thickness compared to wild-type vein grafts. Muto et al also demonstrated that ephrin-b2/fc activated Eph-B4 co-localized with cav-1 and that cav-1 heterozygous knockout mice also demonstrated increased intimal thickening implying that cav-1 is involved in downstream transduction of Eph-B4 activation. Finally, while no definitive mechanism was determined for the reduction in wall thickness with Eph-B4 activation, it was observed that ephrin-b2/fc treatments increased nitric oxide signaling *in vitro* and caused a preservation of Eph-B4 protein within the endothelium of the vein graft *in vivo*.

To further investigate the role of Eph-B4 in vein graft adaptation, endothelial functions were examined in heterozygote knockout mouse lung endothelial cells under the hypothesis that knockout endothelial cells would resemble vein grafts undergoing early adaptation. Theses studies demonstrated that knockout endothelial cells did indeed exhibit less proliferation, migration, and nitric oxide release than wild-type cells while secreting more of the smooth muscle cell mitogens VEGF-A, PDGF-BB, and TGF-β (42). Jadlowiec et al also demonstrated that while VEGF-A upregulation occurred in both the knockout endothelial cells and early vein grafts, subsequent AKT phosphorylation was seen in the endothelial cells

and not whole vein grafts while ERK phosphorylation was seen in the vein grafts and not endothelial cells. These findings seem to suggest that VEGF-A which is also known to downregulate Eph-B4 may have differential effects on smooth muscle and endothelial cells (42, 43).

To summarize, Eph-B4 and its ligand ephrin-B2 are embryologic determinants of venous and arterial fate in the developing vasculature. Eph-B4 which is predominantly, but not exclusively, expressed in the adult venous endothelium is lost during vein graft adaptation. Eph-B4 reduction in the endothelium as simulated with heterozygote knockouts is associated with many of the factors known to be active in vein graft adaptation and neointima formation such as loss of endothelium, loss of nitric oxide secretion, and secretion of smooth muscle growth factors. Finally exogenous Eph-B4 activation with soluble ephrin-B2/Fc can reduce neointimal formation *in vivo* and vein derived from Eph-B4 heterozygote knockout mice leads to an increase in neointima formation.

Specific Aims of this Investigation

With a promising role for Eph-B4 activation in reducing neointimal hyperplasia established in mice, the next step is to determine whether these findings translate to human tissue. Testing of potential therapeutics *in vitro* in human samples is essential not only to ensure that the findings translate across species but also because there exists very real biologic differences between vein graft tissue derived from a mouse and that harvested from a vasculopathic patient requiring bypass surgery. Furthermore, while Eph-B4 activation via ephrin-B2

ligand activation appears an attractive therapeutic target, multiple intra-peritoneal injections in humans as an adjuvant therapy to bypass surgery poses obvious problems such as unanticipated systemic effects, patient discomfort, and increased chance of infection. Thus ,it is necessary to investigate alternative forms of drug delivery to make Eph-B4 activation a more promising target for clinical trials.

Purpose:

To determine whether the reduction in neointimal hyperplasia development in vein grafts with Eph-B4 activation seen in a mouse model will translate to human tissue.

Hypothesis 1: Eph-B4 activation will reduce neointimal hyperplasia in human saphenous vein in vitro

Specific Aim #1: To compare ephrin-b2/fc treated and untreated human saphenous vein rings in an organ culture model of neointimal hyperplasia

Hypothesis 2: Eph-B4 activation causes a reduction in the loss of Eph-B4 during vein graft adaptation

Specific Aim #1: To compare the expression of levels of treated and untreated vein rings to determine if Eph-B4 activation reduces Eph-B4 loss

Hypothesis 3: Eph-B4 signaling in human endothelium will recapitulate findings in mouse endothelium

Specific Aim #1: To compare levels of phosphorylated AKT and ERK in ephrin-b2/fc treated and untreated endothelium

*Specific Aim #2***:** To demonstrate that ephrin-b2/fc activation of Eph-B4 proceeds through a cav-1 dependent manner

Hypothesis 4: Endothelial activation of Eph-B4 is possible with a one time adventitial application of ephrin-B2/Fc to human veins.

Specific Aim #1: To demonstrate endothelial Eph-B4 activation following application of pluronic gel containing ephrin-B2/Fc to the adventitia of a human saphenous vein in an arterial bioreactor

Methods:

Saphenous Vein Organ Culture

Excess saphenous vein remnants were secured from patients undergoing coronary artery bypass grafts (HIC protocol #9908011041) at a large academic medical center following the completion of surgery. They were transported in ice-cold low glucose Dulbecco's Modified Eagle's Medium (Gibco, Carlsbad, CA) and immediately cut into 5-10 millimeters (mm) cross sectional rings. Day 0 samples were immediately fixed in formalin. Additional samples from adjacent sections of the vein were placed in 12 well tissue culture dishes with Roswell Park Memorial Institute (RPMI 1640, Gibco) supplemented with 30% fetal bovine serum, 1% L-glutamine, 1% penicillin/streptomycin for 2 weeks at 37 degrees Celsius (C). In a blinded fashion the media was either supplemented with recombinant human ephrin-B2/Fc chimera (R&D Systems, Minneapolis, MN) at a concentration of 2 micrograms (μg)/ml or not. The media was changed every 2-3 days and samples were fixed in formalin or frozen in optimal cutting temperature compound (Tissue-Tek Sakura Finetek, Torrance, CA).

Histology and Morphometric Measurements

Following formalin fixation samples were transported in 70% ethanol to the Yale Research Histology Laboratory (New Haven, CT) embedded in paraffin, 7 micrometer (μm) sections were cut and slides were stained with Verhoeff-Van Gieson (VVG) elastin stain, hematoxylin and eosin (H&E), or terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). Slides were

examined and multiple pictures were taken per sample. Using ImageJ software (NIH, Bethesda, MD) the area of intima and media were measured in 4 quadrants of the vein ring. Intima was defined as the area on the luminal side of the internal elastic lamina or areas of disordered smooth muscle cell proliferation luminal to the ordered, linear smooth muscle cell layer. The overall area of intima was divided by the area of the media to yield and an intima:media (I:M) ratio.

Quantitative reverse transcriptase polymerase chain reaction

RNA was isolated vein rings by taking OCT embedded samples and collecting approximately 50 10 μm thick sections cut on a microtome. These sections were washed twice with cold, RNAse, DNAse free water to dissolve OCT. The veins were then homogenized by repeatedly aspirating the lysate through a 22 gauge needle in beta-meracptoethanol containing lysis buffer as provided in the RNeasy Mini Kit (Qiagen, Hilden, Germany). The remainder of the isolation and purification steps were carried as specified in the manufacturer's instructions. RNA isolation from cell culture was also carried out according to manufacturer's instructions using the RNeasy Mini Kit. RNA quantification and purity assessment was done using nanodrop spectrophotometer measurement. Reverse transcription was performed using the SuperScript III First-Strand Synthesis Supermix (Invitrogen, Carlsbad, CA). Real-time quantitative . Real-time quantitative PCR was performed using SYBR Green Supermix and the iQ5 Real-Time PCR Detection system (Bio-Rad Laboratories, Hercules, CA).

Primers for specific genes are presented in table 1. Primers were designed using publicly available online software and oligos were synthesized by the Keck Oligo Synthesis Facility (New Haven, CT). Correct target size amplification and exclusion of nonspecific amplification was confirmed by performing electrophoresis on amplification products on 2% agarose gel as well as performing melt curve analysis. For analysis reactions were normalized to GAPDH as a housekeeping gene.

Gene		Sequence
GAPDH	5'->3'	GAGAAGGCTGGGGCTCATTT
	3'->5'	AGTGATGGCATGGACTGTGG
Eph-B4	5'->3'	ATGCCCGTCATGATTCTCAC
	3'->5'	GGAAAGGCCAAAGTCAGACA
Ephrin-B2	5'->3'	AACTGTGCCAAACCAGACCA
	3'->5'	GCAGAACTTGCATCTTGTCCA
Osteopontin (OPN)	5'->3'	CTGCCAGCAACCGAAGTTTT
	3'->5'	TCCTCGCTTTCCATGTGTGA

Table 1. Primers employed in qPCR reactions.

Human Umbilical Venous Endothelial Cell (HUVEC) culture

HUVEC cells were cultured in Endothelial Basal Medium with Bulletkit growth factors (Lonza, Basel, Switzerland) supplemented with 15% fetal bovine serum and 1% penicillin/streptomycin. Cells were grown on either glass coverslips or 6 well plates coated with 0.1% gelatin. Cells were assayed between passages 2-5.

Prior to stimulation, growth medium was removed and cells were placed in serum free starvation media for at least 3 hours.

Western blot and immunoprecipitation

Ephrin-b2/fc was pre-clustered with human IgG/Fc (Sigma Aldrich, St. Louis, MO) at a 5:1 molar ratio for 20 minutes at 37 C and then cells were treated with the clustered ligand. Following stimulation, cells were washed with ice cold PBS and then lysed using a RIPA buffer supplemented with protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO) and phosphatase inhibitors (Thermo Scientific, Waltham, MA). Protein concentrations were assessed using a colorimetric assay (Bio-rad, Hercules, CA) and then matching amounts of protein were run on a SDS-PAGE gel and then transferred to a nitrocellulose membrane. Following blocking with 2% bovine serum albumin, membranes were probed overnight with primary antibodies listed at the end of this section. Membranes were developed using Supersignal West Pico kit (Thermo Scientific, Waltham, MA). Immunoprecipitation was performed by incubating at least 200 µg of protein with a phosphor-tyrosine antibody bound sepharose bead overnight and then western blot conducted as above.

Con-focal and immunofluorescence microscopy

HUVEC cells were plated on glass coverslips, serum starved, and treated. They were fixed in 10% neutral buffered formalin for 15 minutes, washed, and the cell membranes were made permeable by brief treatment with triton-x-100 and

then blocked with goat serum and exposed to primary antibody overnight. They were treated with alexa-flouro 488, 568, and 647 secondary antibodies and mounted with a DAPI containing media. They were examined using AxioImager A1 (Carl Zeiss Inc, Oberkochen, Germany) or a TCS SP5 II (Leica, Solms, Germany). Vein samples undergoing immunofluorescence were fixed with formalin and then embedded in paraffin and cut into 5 μm sections and placed on slides. Following deparaffinization, antigen retrieval was conducted by boiling with 10mM citric acid with 0.05 tween pH 6 for 20 minutes. Blocking was conducted with goat serum and primary antibody was applied overnight. Secondary antibodies were applied as above. Autofluoresence Elliminator Reagent (Millipore, Billerica, MA) was used and then sections were mounted in DAPI containing mounting media and imaged on an AxioImager A1 (Carl Zeiss Inc, Oberkochen, Germany)

Primary antibodies to the following antigens were obtained as follows: Eph-B4 catalog #64820, Caveolin-1 catalog #17052 were obtained from Abcam (Cambridge, England). Antibodies to phosphorylated AKT ser 473 Catalog #9271, phosphorylated ENOS ser 1177 catalog #9571, GAPDH catalog #2118, phosphorylated-tyrosine conjugated to sepharose beads Catalog #9419 were obtained from Cell Signaling Technologies (Danvers, Ma). Antibody to phosphor-tyrosine residues catalog #05-1050 was obtained from Millipore (Billerica, MA). Antibody to total ENOS catalog #BD610296 and phosphorylated cav-1 (tyr14) #BD611338 were obtained from BD Biosciences (Franklin Lakes, NJ).

Pluronic Gel experiments:

Ephrin-B2/Fc was dissolved in a 30% pluronic gel (Sigma-Aldrich, St Louis, MO). Excess saphenous vein was taken from the OR in ice-cold low glucose DMEM and the vein was placed in the Bioreactor (pictured in Appendix A) set up consisting of a glass chamber (fabricated at the Yale Scientific Glass Shop, New Haven, CT) with vein section attached to glass connector which were connected to a glass compliance chamber on either end to equalize pressure in the system, and an intake 0.22μm air filter on the distal chamber. A digital programmable roller-pump (Cole Parmer, Vernon Hills, IL) was used to push media along the system which was placed in a 37ºC incubator. Intraluminal fluid consisted of Endothelial Growth Medium (EGM-2 Lonza, Basel, Switzerland) thickened with Xanthum Gum (sigma) to a viscosity of 3.8 cP; . XG solution was autoclaved to assure sterility before composition with media base. Extraluminal fluid consisted of DMEM supplemented with 30% fetal bovine serum. Specimens were harvested starting approximately 1 cm from the origin of the attached connector in order to avoid areas of turbulent flow at this transition point.

Statistics:

Statistical analysis including one way ANOVA and paired T test were performed using GraphPad version 6.0a for MAC OS X, GraphPad Software (La Jolla, CA). A p value of less than 0.05 was considered significant. Means are reported as arithmetic means with standard errors of the means following.

Results:

Validating unbiased method of measuring intima:media ratios.

The development of neointimal hyperplasia can be irregular around the luminal surface of a vessel. In previous studies using intima:media ratios to measure the development of neointimal hyperplasia, linear measurements of the intima and media have been used. Various schemes have been used to place the exact points of measurement in an unbiased manner such as 8 equally spaced radial measurements. However, there is still the chance of inadvertent bias to affect the process since placing the exact point of measurement requires the observer to draw two lines on a computer screen. The placement of these lines can drastically affect the measurement. Moreover when the vein is not a perfect circle radial distribution of the lines is not straightforward. Therefore we investigated whether measurements of the area of the intima and the media could be more comprehensive and reduce the chance of bias.

Vein rings from three donors were put in organ culture for 14 days. Two strategies for measuring the intima:media ratio were employed. Four equally spaced radial lines were drawn to designate the points to measure the linear intima and media lengths (Fig 1a). On the same sample four quadrants were designated and then total area of the intima and media were measured in these quadrants as outlined by the lines (Fig 1b). Using both the linear and area methods of measuring mean intima:media ratios increased over time in the same samples (Fig 1 c,d). Mean intima:media ratios increased over time in both the linear and area measurements by approximately the same amount (Fig 1 e). Finally the percent change in

intima:media ratio from day 0 was similar between both methods for individual samples differing by only 20.6% ± 1.4% (Fig 1 f). Given the similar results both as a group and with each individual sample between the two methods we concluded that the two results were comparable. With the smaller chance for any inadvertent bias in the area method, we decided to proceed with the area strategy and as well to blind the observer.

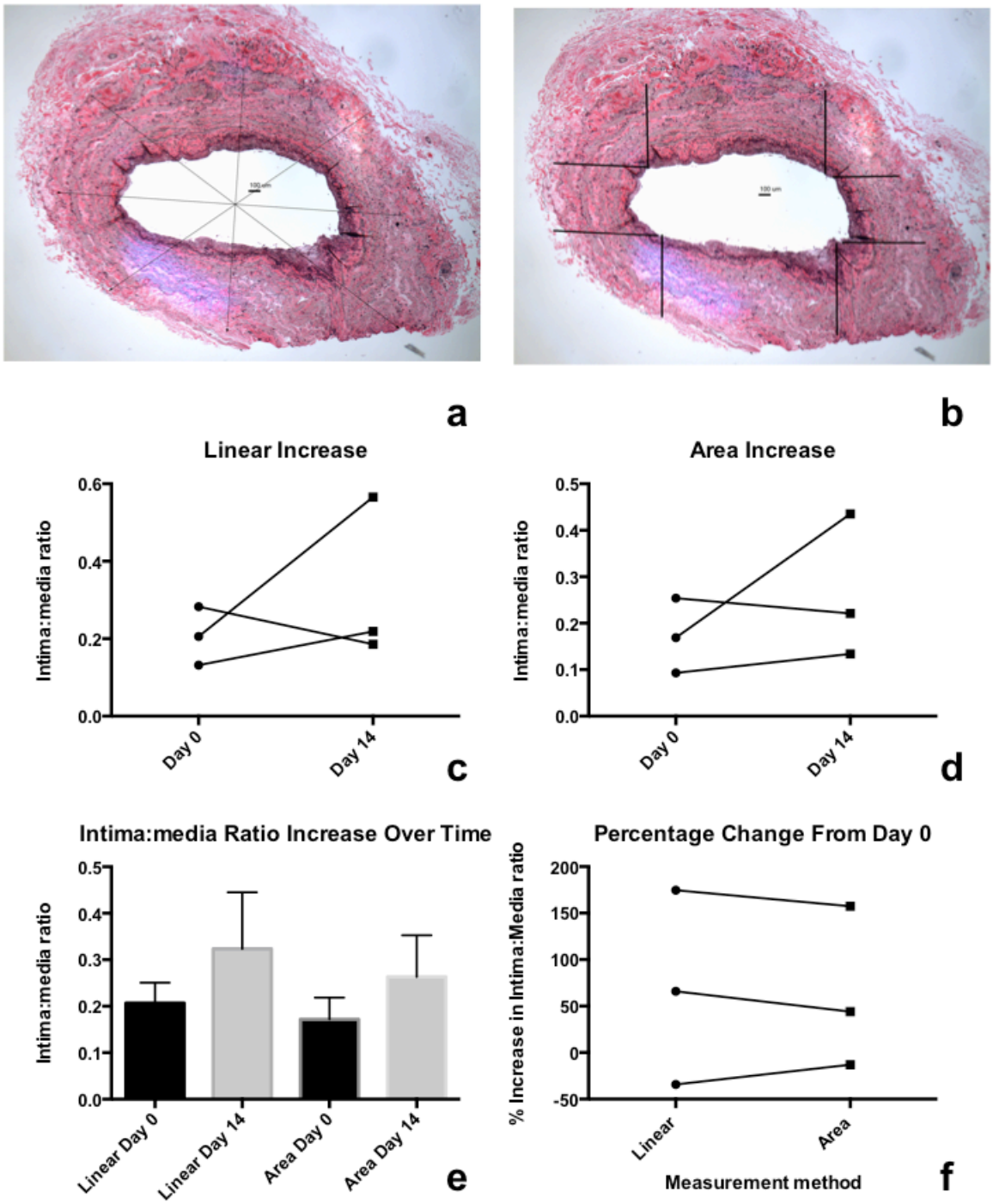

Figure 1. Comparison of linear versus area measurement method for determining intima:media ratios. Depiction of linear (a) and area (b) methods. Individual sample change in intima:media ratio change by linear (c) and area (d) methods. Average increase in intima:media ratios over time (e). Comparing individual percent change from day 0 for each method (f).

Hypothesis #1: Treatment of saphenous vein rings with soluble ephrin-B2/Fc will decrease the development of neointimal hyperplasia in organ culture.

To examine the effect of exogenous Eph-B4 stimulation on the development of neointimal hyperplasia in human saphenous vein, vein rings from the same donor and adjacent section of vein were placed in organ culture for 14 days with or without soluble Ephrin-B2/Fc the activating ligand for Eph-B4. Given the variability in the size and pre-existing intimal hyperplasia pre-culture, intima:media (I:M) ratios were used to measure the development of the neointimal hyperplasia in the vein rings. The mean baseline I:M ratio was 0.456 ± 0.097 (n=19). Over 14 days in organ culture this ratio increased to 0.726 ± 0.142 (n=19) in untreated veins and 0.630 ± 0.132 (n=19) (Fig 2c). Using paired T-tests, these results demonstrated a significant difference in I:M ratios between the matched controls in the treated and untreated vein rings after 14 days in organ culture (Fig 2c). There was a significant increase in I:M ratios between the baseline group and both treated and untreated day group. The mean individual percentage increase in intima:media ratio from day 0 to day 14 was 78.43 ± 23.34% for untreated veins and 53.09 ± 21.40% for treated veins (Fig 2e) .Examination of individual matched controls revealed variability in the response to treatment. 13 of 19 matched pairs saw a reduction in the increase of I:M ratio over time in the treatment group, however, six of the nineteen treated rings demonstrated no change or even a slight increase in I:M ratio between the treated and untreated vein rings (Fig 2 d).

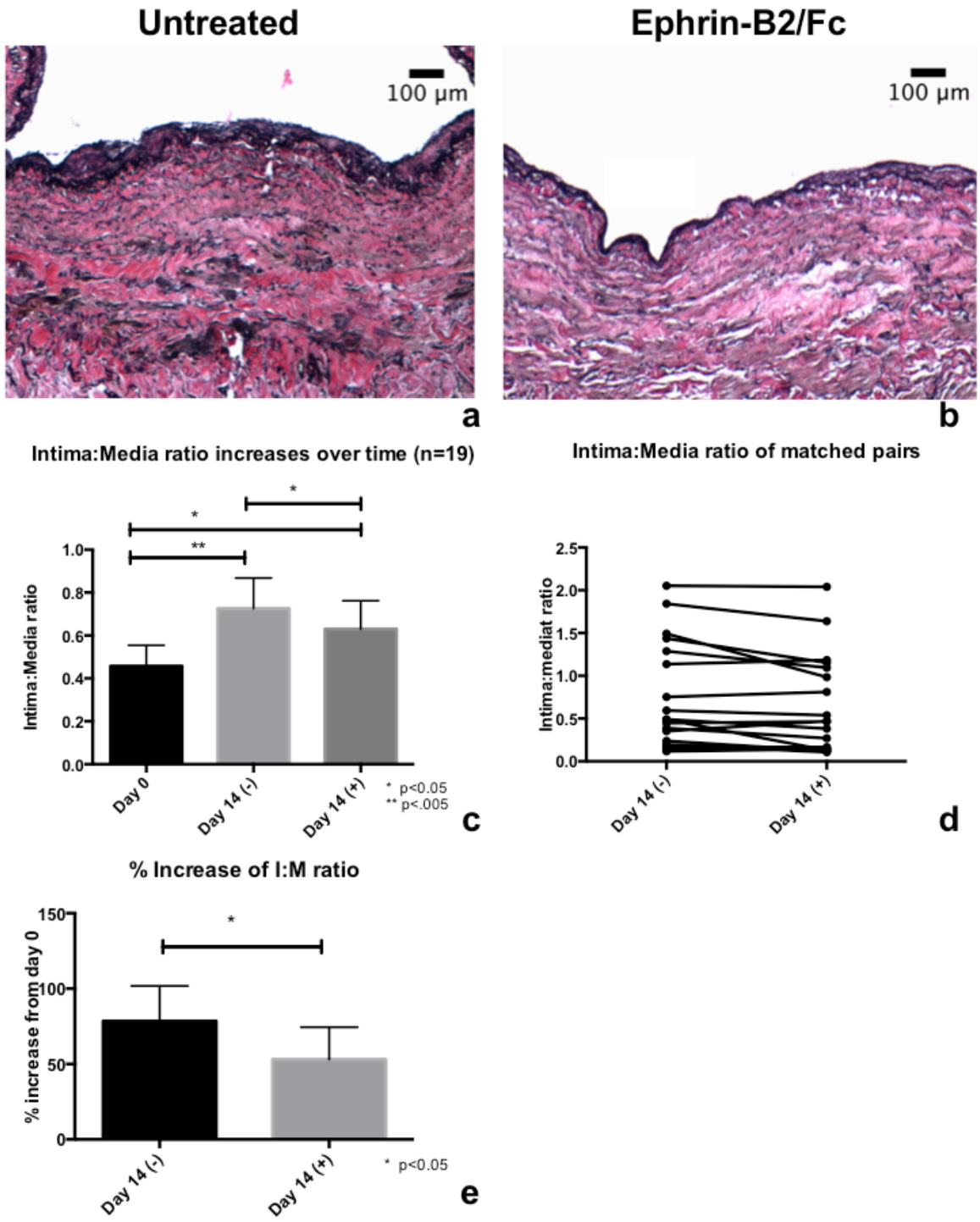

Figure 2. Ephrin-B2/Fc treatment reduces intima:media ratios in saphenous vein organ culture. Neointima formation in untreated (a) and treated (b) vein rings. The average intima:media ratios for baseline, untreated, and treated vein rings (c). Treated and untreated samples matched pairs compared at day 14 (d). Percent increase of intima:media ratios from baseline for treated and untreated veins (e).

Hypothesis #2: Treatment of saphenous vein rings in culture with exogenous ephrin-B2/Fc will preserve Eph-B4 expression levels.

To examine whether Eph-B4 expression levels would be preserved in ephrin-B2/Fc treated vein rings quantitative polymerase chain reaction (qPCR) assays were performed on vein ring lysates. Consistent with previous *in vivo* results, Eph-B4 and ephrin-B2 mRNA expression fell over time in organ culture (Fig 3). There was however no significant difference in Eph-B4 levels in the ephrin-B2/Fc treated and untreated vein rings. To demonstrate that the loss of Eph-B4 and ephrin-B2 gene expression did not represent a global loss of mRNA production, osteopontin levels were examined. Osteoponin levels increased in organ culture over time and there was no difference between treatment and non-treatment groups.

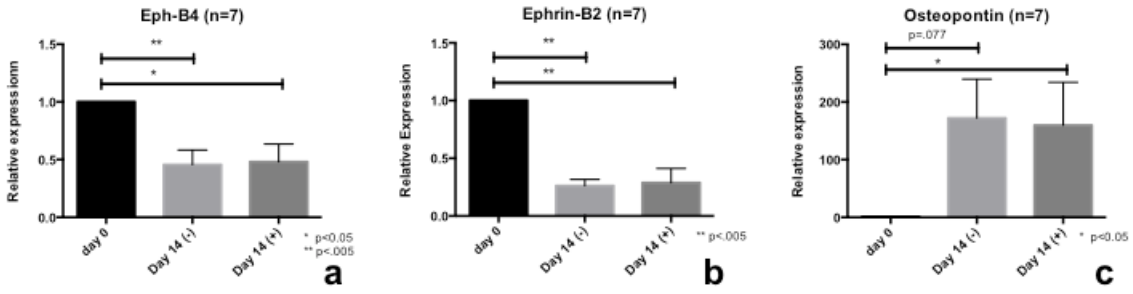

Figure 3. Gene transcription levels of Eph-B4, ephrin-B2, osteopontin in saphenous vein culture mimic *in vivo* results. Eph-B4 (a), ephrin-B2 (b), osteopontin (c) gene transcription levels of baseline, treated, and untreated vein rings.

Hypothesis #3: Treatment of endothelial cells in culture with exogenous ephrin-B2/Fc will cause increase in Eph-B4 expression.

Having not found any difference in reduction of Eph-B4 expression in the ephrin-B2/Fc treated vein rings, we hypothesized that since the majority of the mass and thus mRNA of the vein rings was smooth muscle cell and adventitia and

not endothelium after 14 days in organ culture some ephrin-B2/Fc induced endothelial cell specific gene expression changes may have been obscured. To determine if this was the case Human Umbilical Venous Endothelial Cells (HUVECs) were treated with ephrin-B2/Fc and their Eph-B4 gene expression levels were assessed (Fig 4). There was no change in Eph-B4 expression between the control and treatment groups.

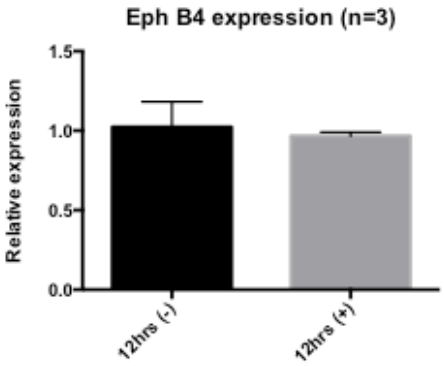

Figure 4. Ephrin-B2/Fc treatment does not increase Eph-B4 expression in HUVEC cells.

Hypothesis #4: Ephrin-B2/Fc treatment of HUVECs will lead to Eph-B4 phosphorylation and downstream signaling changes in AKT, cav-1, and ENOS.

Since ephrin-B2/Fc did not alter Eph-B4 transcription levels, we sought to assay its effect on Eph-B4 phosphorylation and other downstream signaling cascades in endothelial cells. As expected ephrin-B2/Fc treatments caused a strong increase in Eph-B4 phosphorylation in HUVECs cells (Fig 5a). Ephrin-B2/Fc treatments also caused an increase in phosphorylated AKT (ser473) and cav-1 (tyr14) over time (Fig 5b, c). Phosphorylated ENOS (ser1177) expression also appeared to be upregulated although not to the degree of AKT or cav-1. Using con-

focal microscopy we observed that ephrin-B2/Fc treatments caused an increase in co-localization between caveolin-1 and Eph-B4 confirming previous observations made in mice (Fig 6).

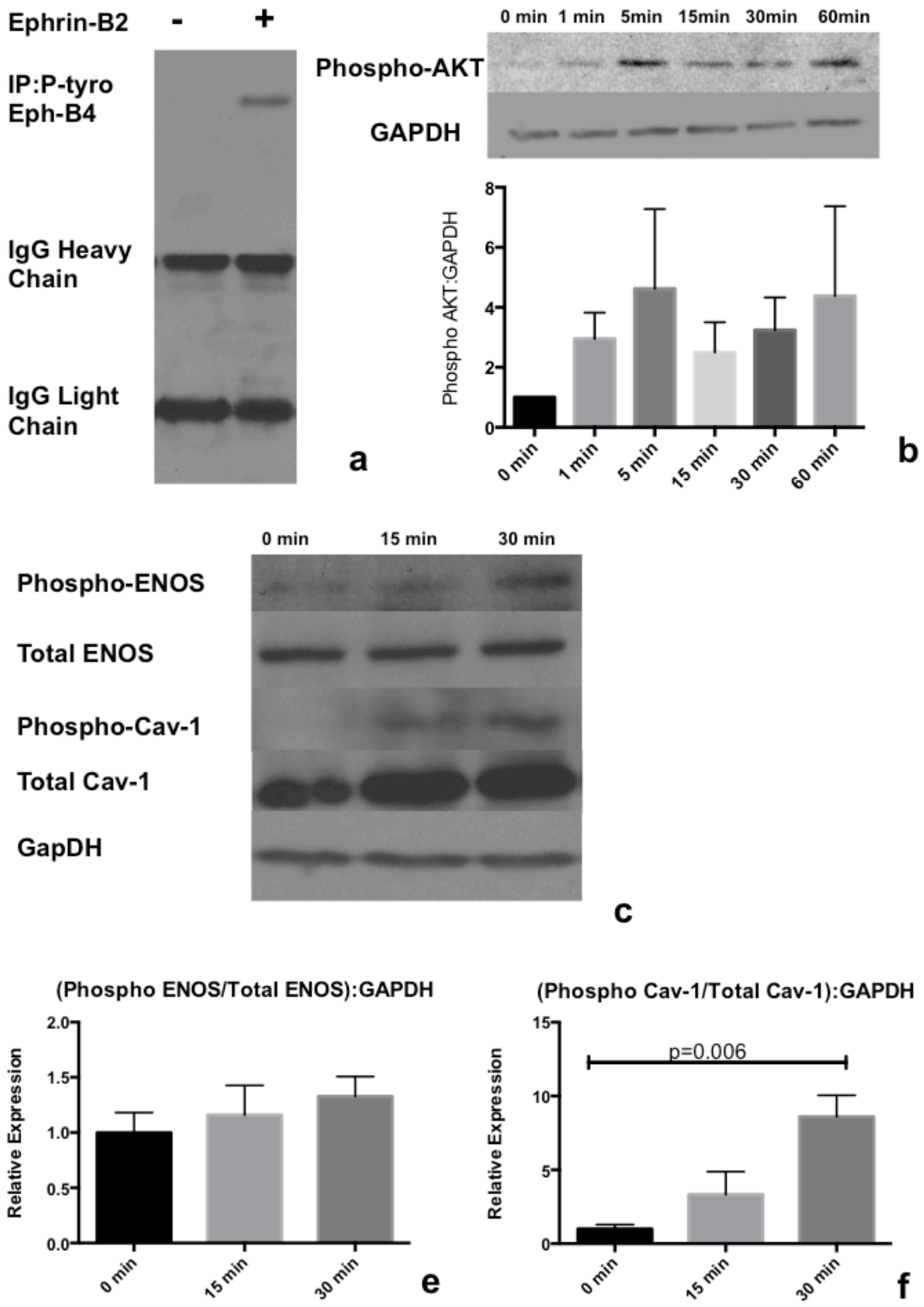

Figure 5. Ephrin-B2/Fc treatment of HUVEC leads to Eph-B4 phosphorylation and downstream signaling changes. Immunoprecipitation of HUVEC treated with ephrin-b2/Fc: initial pull down with phospho-tyrosine and then probed for Eph-B4 (a).

Ephrin-B2/Fc treated cells demonstrating increase of phosphorylated AKT (ser473) over time (b). HUVEC cells treated with ephrin-b2/fc assessed for phosphorylated ENOS (ser1177) and Cav-1 (tyr14) (c). Quantification of expression of phospho-ENOS (d) and cav-1 (e).

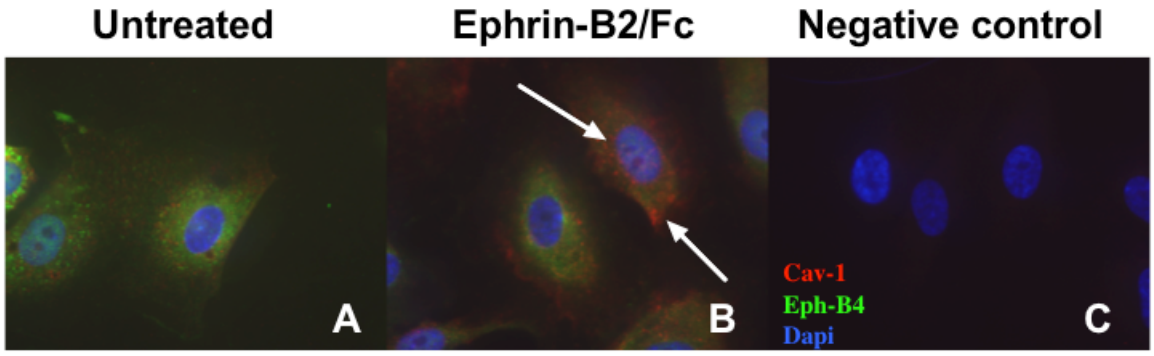

Figure 6. Ephrin-B2/Fc treatment causes co-localization of Cav-1 and Eph-B4 in HUVEC cells. Untreated HUVEC cells (a). Ephrin-B2/Fc treated cells demonstrating co-localization of Eph-B4 and cav-1 (b). Negative control cells without primary antibody (c).

Hypothesis #5: Endothelial Eph-B4 activation can be achieved with application of ephrin-B2/Fc dissolved in a pluronic gel applied to the adventitia of the vein.

Saphenous vein was placed in a bioreactor that simulated arterial flow. Prior to initiation of flow the adventitia of the vein was treated with pluronic gel containing ephrin-B2/Fc or not. The gel was allowed to adhere to the adventitia of the vein and then arterial flow was run for 24 hours. At the end of 24 hours the treated veins showed an increase in co-localization of Eph-B4 and phospho-tyrosine residues especially in the endothelium of the vein that were not present either at time 0 or in the untreated vein (Fig 7).

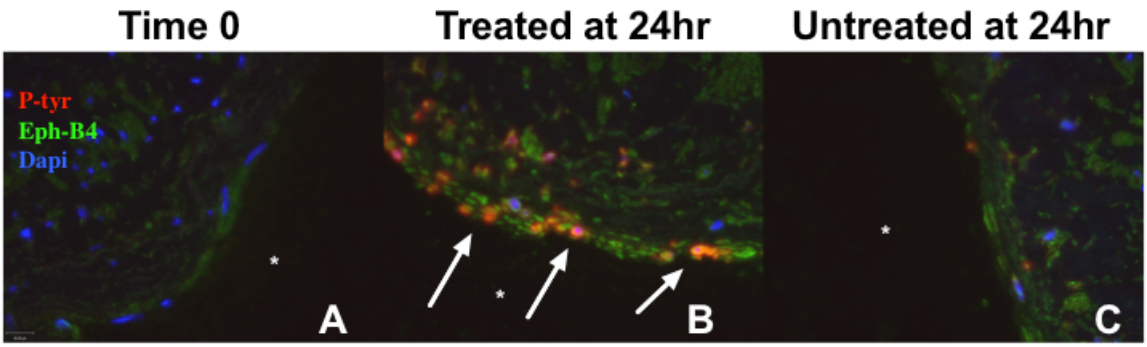

Figure 7. Adventitial delivery of ephrin-B2/Fc causes eph-B4 phosphorylation. Saphenous vein at time 0 (a). Saphenous vein treated with ephrin-b2/fc at 24hrs with increased co-localization of eph-b4 and phosphor-tyrosine (b). Untreated saphenous vein (c). White asterisks mark lumen of the vessel.

Discussion:

Recent work in mouse models demonstrated that Eph-B4 stimulation with ephrin-B2/fc was effective in reducing intimal thickness during vein graft adaptation (41). Thus, while Eph-B4 activation appears to be a promising therapeutic target for reducing neointimal hyperplasia in vein grafts, it was necessary to determine whether these findings in mice translated to human tissue. Unfortunately, there are few established models to simulate vein graft adaptation *in vitro* with human tissue. Since we ultimately wanted to evaluate the effect of Eph-B4 stimulation on intimal hyperplasia over time, the saphenous vein organ culture model was chosen, because it yields a histologic phenotype from which intimal hyperplasia can be measured by tracking intima:media ratios.

Saphenous vein rings incubated with soluble ephrin-B2/fc developed less intimal hyperplasia over 14 days than rings from the same donor and adjacent section of vein (Fig 2c). The reduction in development of intimal hyperplasia is consistent with experiments performed in mice using large systemic injections of the ligand (41). The reduction in I:M ratios was not a universal effect as close to 1/3 of the treated vein rings did not demonstrate a decrease in intimal hyperplasia compared to untreated controls (Fig 2d). When employing human samples it is not surprising that there are variable responses to treatment. One of the strengths of this study is that these experiments were performed on saphenous veins from patients undergoing bypass surgery. Thus, unlike animal models or tissue from healthy volunteers, these veins are representative of the actual tissue from vasculopathic patients who require saphenous vein grafts.

Given the ethical and clinical restraints of securing human tissue, the saphenous vein used in these experiments was not procured until the operation was completed. It is well documented that surgical trauma, ischemic time, vein quality and even vein harvesting technique can affect vein grafts in both *in vitro* assays of relaxation, reactive oxygen species (ROS) generation, and even histologic appearance, as well as their *in vivo* patency rates (44-47). Accordingly, a degree of variability is inherent in this experimental procedure. It is unknown how initial vein characteristics such as extent of endothelial denudation would affect the action of prolonged Eph-B4 stimulation. However since it is known that the majority of Eph-B4 expression and thus hypothesized site of action is in the endothelium the effect of endothelial damage on efficacy of Eph-B4 activation may warrant further investigation.

While eprhin-b2/fc Eph-B4 activation has been shown to affect multiple endothelial processes including nitric oxide production, migration, and proliferation as well as interact with AKT and MAPK signaling networks there is not yet a definitive mechanism for its role in reducing intimal hyperplasia (42, 48, 49). In mouse vein grafts, eprhin-b2/fc stimulation seem to preserve Eph-B4 protein in the endothelium of the vein grafts (41). Thus, we hypothesized that ephrin-b2/fc treatment of saphenous vein rings would preserve Eph-B4 expression, which is normally lost vein graft adaptation. Using qPCR we examined Eph-B4 expression in treated and untreated saphenous vein rings in organ culture (Fig 3a). While both Eph-B4 and ephrin-B2 expression levels fell during organ culture there was no difference in the treated and untreated groups (Fig 3a,b). The loss of Eph-B4 and

ephrin-B2 during organ culture corresponded with previous results from mouse, rat, and human vein grafts (21, 41). To ensure that the reduction in Eph-B4 and ephrin-b2 transcription levels was not due to a global reduction of gene transcription in organ culture we also tested osteopontin expression, a marker of venous adaptation, and found that it increased over time (Fig 3c) (50).

Since the media and adventitia make up the vast majority of the cellular mass of the vein rings in organ culture, our qPCR results most likely represented mRNA transcription within smooth muscle cells and fibroblasts. Since previous studies have shown the majority of Eph-B4 is expressed in the endothelium, we wanted to more closely examine the effects of ephrin-b2/fc on the endothelium, and thus employed a HUVEC cell line culture to determine whether ephrin-b2/fc increased Eph-B4 expression in endothelial cells. HUVEC cells were treated with ephrin-b2/fc and Eph-B4 gene expression was assayed using qPCR, however, there was no increase in Eph-B4 expression was observed (Fig 4).

While ephrin-b2/fc treatments have been shown to increase proliferation in mouse endothelial cell lines, and Eph-B4 knockout cells have demonstrated reduced proliferation, this does not necessarily require upregulation of Eph-B4 expression. The preservation of Eph-B4 expression in the endothelium of ephrin-b2/fc treated vein grafts may represent overall endothelial recovery, and thus endothelial Eph-B4 expression may just be a marker of the underlying processes contributing to the reduction in intimal thickness rather than the driving mechanism. Therefore Eph-B4 stimulation of endothelial cells does not necessarily need to lead to increased Eph-B4 mRNA expression levels for Eph-B4 to be retained in the wall of vein grafts.

Since ephrin-B2/fc treatments of saphenous vein ring in organ culture yielded a histologic phenotype that was consistent to previous studies, we sought to determine whether intracellular signaling findings associated with Eph-B4 activation in mice also translated into human tissues. As expected ephrin-B2/fc treatment of HUVEC cells led to Eph-B4 phosphorylation (Fig 5a). Ephrin-B2/fc treatment also lead to increased AKT phosphorylation (Fig 5b). This finding is consistent with previous experiments using human microvascular endothelial cells isolated from the bowel. These endothelial cells demonstrated increased proliferation and nitric oxide production in an AKT dependent manner in response to ephrin-b2/fc treatment (49). Given these findings we also sought to evaluate ENOS phosphorylation which appeared to be upregulated with ephrin-b2/fc treatment, however, not significantly so when the levels were quantified.

Increased nitric oxide production is one likely possible mechanism for ephrin-b2/fc treatment reduction of intimal hyperplasia. Inducible nitric oxide transfection of porcine vein grafts has been shown to decrease both intimal hyperplasia and reduce overall cellular proliferation (51). While our results appear to support this in that ephrin-b2/fc treatments appeared to cause increases in AKT and ENOS phosphorylation, statistical significance was not reached with the densitometry. It is possible that HUVECs demonstrate a less dramatic response to ephrin-b2/fc treatment than adult endothelial cells although endothelial AKT phosphorylation has been shown to cause eNOS phosphorylation and increased nitric oxide release in HUVECs cells before (52). Thus while ephrin-b2/fc induced AKT and ENOS phosphorylation in human cells is a possible mechanism for the

phenotype observed more work is needed to confirm this. Experiments assessing direct nitric oxide release in response to Eph-B4 stimulation are in process. Interestingly AKT inhibition via PTEN overexpression in a viral vector also lead to reduction of intimal hyperplasia in a canine model of coronary vein grafting (30). However this was likely due to inhibition of AKT mediated proliferation in smooth muscle cells.

Previous mouse experiments demonstrated that ephrin-b2/fc treatment caused Eph-B4 to associate with caveolin-1, a major caveolae coat protein critical in caveolae assembly and leads to caveolin-1 phosphorylation (41). Using microscopy we confirmed that ephrin-b2/fc treatment causes co-localization of Eph-B4 with cav-1 in HUVECs as well (Fig 6a). The increase in cav-1 phosphorylation seen with western blotting following ephrin-b2/fc treatment further strengthened the association between Eph-B4 activation and downstream caveolin signaling (Fig 5c,e) *In vivo* confirmation of this comes from Muto et al who demonstrated that loss of cav-1 eliminates the ephrin-B2/fc intimal hyperplasia reduction in mouse vein grafts. This experiment demonstrated the importance of cav-1 as an intermediate in Eph-B4 function in the vein graft. Caveolae are small cellular vesicles that along with their associated caveolin coat proteins have been shown to play important roles in signal transduction especially in endothelial cells which are caveolae rich (53). Interestingly caveolin-1 knockout endothelial cells show an increase in nitric oxide production (54). Thus ephrin-b2/fc activation of Eph-B4 may reduce intimal hyperplasia in a separate nitric oxide independent manner as well.

Systemic dosing of mouse vein graft recipients with ephrin-B2/Fc led to a reduction in intimal thickness (41). Similarly treatment of saphenous vein rings with eprhin-B2/Fc dissolved in the organ culture media also lead to the desired reduction in intima:media ratios. However we sought to demonstrate that Eph-B4 activation was possible with an adventitial dosing of ephrin-B2/Fc dissolved in a pluronic gel. Using immunofluorescence microscopy we showed that Eph-B4 and phospho-tyrosine co-localized primarily in the endothelium of the saphenous vein was possible after 24 hours in an arterial flow simulator indicating Eph-B4 activation (Fig 7).

Local drug delivery can be advantageous to the bypass recipient. Drugs delivered locally can be given at high dosages and minimize systemic effects. Adventitial delivery of drugs can avoid further endothelial disruption. By necessity bypass surgery exposes the anatomic sites especially the distal anastomosis where subsequent stenosis is most likely to occur. Using hydrogels such as pluronic gel to deliver drugs in experimental models has increased in recent years, however, hydrogels tend to elute dissolved compounds in several days and thus the intended drug effect may be transient and unable to durably affect neointimal hyperplasia. which can develop over months to years (55). A limitation to our findings is that we examined Eph-B4 activation after only 24 hours in the arterial bioreactor since this length of time we have validated our bioreactor for (unpublished data). Within the last year, *ex vivo* human saphenous vein models have been developed that extend this time to 1 week and testing ephrin-b2/Fc stimulation in this model is a logical next step (56). Numerous labs including this one have employed viral vectors to

increase desired gene expression to inhibit intimal hyperplasia (21, 32, 41, 51). While gene therapy for human treatment remains controversial, there are multiple future directions to explore in providing local but durable Eph-B4 activation as a potential adjunctive therapy (57).

There are several limitations to the saphenous vein organ culture model used in these experiments. A vein ring in organ culture is not subject to flow, shear stress, or other hydrostatic forces that have been shown to affect the development of intimal hyperplasia and thus Eph-B4 interaction with these pathways cannot be assessed in this model (23, 58). There is no site of anastomosis, which is a common site of intimal hyperplasia development and restenosis (59). Vein rings in organ culture cannot be infiltrated by circulating immune cells or graft extrinsic cells which have been shown to contribute to vein graft remodeling (60). Despite these limitations, saphenous vein organ culture has been employed in the scientific literature since 1990 (61). The model offers reliable replication of cellular and extracellular components seen in neointimal hyperplasia in vein grafts (62). In recent years, an immune deficient mouse has been grafted with human arterial tissue and injected with human immune cells as a model to study immune system interaction with ischemic reperfusion injury(63). An extension of this model using human saphenous vein and an immune deficient rat is an attractive future direction to study Eph-B4 activation over longer time courses, Eph-B4s interactions with host immunity and infiltrating cells, and different drug delivery methods.

While much of the investigation of Eph-B4 signaling and activation has focused on endothelial cells, it is clear that Eph-B4 is not expressed solely in

endothelial cells and Eph-B4 activation likely affects all cell types in all 3 layers of the vessel wall. Saphenous vein ring endothelial dependent relaxation can be tested *in vitro* following treatment with a drug and given ephrin-B2/Fc treatment to induce nitric oxide release this represents a promising future direction for our research (64). Also multiple techniques exist for smooth muscle cell and endothelial cell co-culture to investigate the interactions between cell types in the vessel wall (65). Given the evidence that Eph-B4 activation can increase endothelial cell migration and proliferation, it would be interesting to see whether ephrin-B2/Fc stimulation could attenuate secreted smooth muscle growth factors during endothelial cell stress and damage. The role of extracellular matrix deposition and its effects on the biophysical properties of vein grafts is an important aspect of vein graft adaptation and long term patency *in vivo* (23). Finally while the current investigation has shown that many of the findings in animal models that made Eph-B4 activation a promising therapeutic target to reduce intimal hyperplasia in vein grafts translate into human tissue, large animal studies over longer periods of time are the critical next step in developing ephrin-b2/Fc as an adjunctive therapy to bypass surgery.

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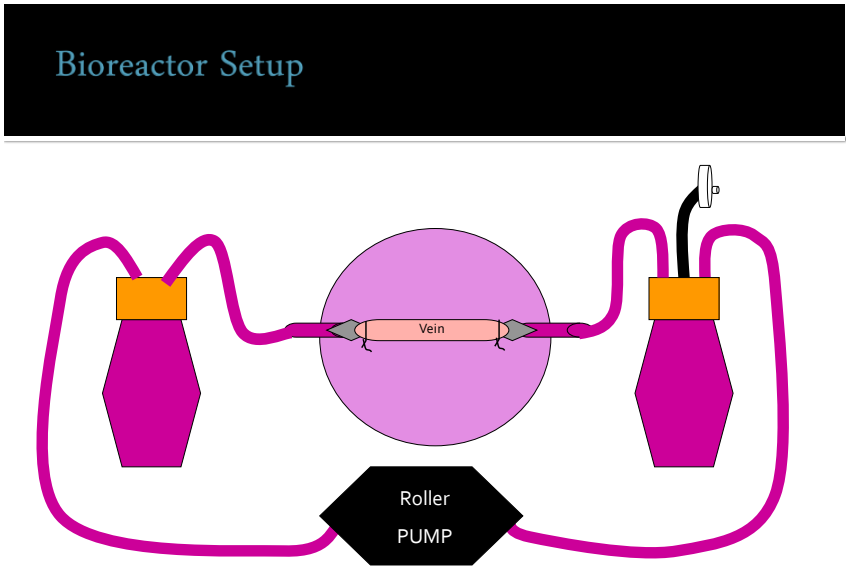
Appendix



a

b

Appendix A: Schematic representation of bioreactor set up (a). Vein is submerged in extraluminal fluid in glass container on right. It is connected to flow which is regulated by programmable pump on left. Flow passes through compliance chambers pre and post vein. Photo of bioreactor set up in incubator (b) Photo courtesy of Lynn Model MD (66)