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Mechanism For Eph-B4 Regulation Of Vein Graft Adaptation

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By
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ABSTRACT

Introduction

Vein graft adaptation to the arterial circulation entails many changes at the cellular/molecular levels, including both cell proliferation and extracellular matrix (ECM) deposition/remodeling, resulting in thickening of the vein wall, and in excessive cases, pathological neointimal hyperplasia. Eph-B4 is a receptor tyrosine kinase and embryonic determinant of veins that, when stimulated, has been shown by our lab to decrease vein graft wall thickening. We hypothesize that among other mechanisms, Eph-B4 exerts this effect through modulating extracellular matrix production and/or degradation, and that Eph-B4 interacts directly with eNOS to mediate some of its downstream effects.

Methods

Mouse Embryonic Fibroblasts (MEFs) were stimulated with 2 ug/ml Ephrin-B2/Fc, the stimulatory ligand for Eph-B4. To determine mRNA changes, cell lysates were collected after various stimulation periods, followed by mRNA purification and RT-PCR for each specified gene of interest. Similarly, mRNA was also extracted from human saphenous veins in culture. For Western Blot and Zymography, both cell lysates and conditioned media (secreted proteins) were collected after various periods of stimulation. *In vivo*, 4 week old vein graft sections from WT and Eph-B4 knockout mice, or from Ephrin-B2/Fc stimulated or unstimulated mice, were stained with Masson's Trichrome, and the total area of blue staining surrounding the vein graft was quantified to determine graft wall collagen content. For eNOS experiments, either Eph-B4 or Y774F

mutant Eph-B4 was transfected into COS cells, followed by Ephrin-B2/Fc stimulation for 1-60 minutes and determination of eNOS phosphorylation utilizing Western Blot.

Results

mRNA expression of Collagen I, Collagen III, Matrix Metalloproteinase 2 (MMP2), MMP9, MMP14, elastin, Tissue Inhibitor of Metalloproteinases 1 (TIMP1), and TIMP2 were not affected by Eph-B4 stimulation. mRNA expression of Collagen 1 in human saphenous veins in culture was not affected by Eph-B4 stimulation. Eph-B4 had no effect on MMP2 and MMP9 cellular protein levels as detected by Western Blot, and no effect on MMP2 protein activity as detected by zymography in cell lysates and conditioned media. Masson's Trichrome staining of mice vein graft sections revealed that most of the collagen within the vein graft was within the adventitia. There were no differences in either collagen content or lumen circumference between vein grafts from WT and Eph-B4 KO mice, and between control mice and mice stimulated with Ephrin-B2/Fc. Dual transfection of cells with endothelial nitric oxide synthase (eNOS) and Eph-B4 resulted in increased eNOS phosphorylation; Eph-B4 stimulation further increased eNOS phosphorylation. Y774 is not crucial to Eph-B4's effects on eNOS.

Conclusions

Eph-B4 likely does not exert its effects on decreasing vein graft thickening through directly modulating ECM deposition and remodeling. Eph-B4 likely exerts its effects through other mechanisms such as modulating cellular proliferation within the

vein graft wall. Eph-B4 likely mediates at least some of its downstream effects through a direct interaction with eNOS.

ACKNOWLEDGEMENTS

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INTRODUCTION

Vein Graft Adaptation

Despite significant advancements in medical knowledge over the recent years, cardiovascular disease remains the leading cause of death in the United States. For advanced cardiovascular disease, bypass surgery with autologous vein is a common and important treatment, both peripherally for peripheral vascular disease and centrally for coronary artery disease (1, 2). However, the adaptation of the vein to this new arterial hemodynamic environment, which includes increased flow, pressures, and oxygen tension, is still not well understood. Successful adaptation plays a crucial role in the long-term function and patency of the vein graft and is characterized by venous dilation and wall thickening. On the other hand, unsuccessful adaptation may involve insufficient or excessive occurrence of these same processes, resulting in thrombosis, stenosis, subsequent patient morbidity, and significant healthcare costs (3, 4).

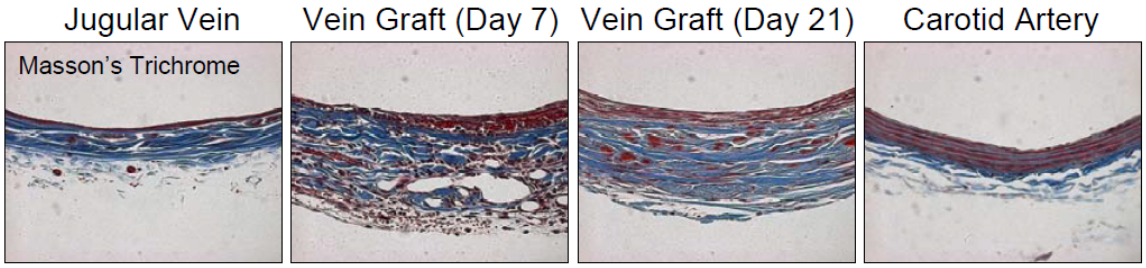


Figure 1. Previous work done by our lab demonstrating extracellular matrix deposition within Trichrome stains of rat vein grafts. (1)

Long-term failure rates for vein grafts are significant, with 1 year primary patency rates reported to range from 80% to as low as 60% (2, 5-7). The most common mechanism of vein graft failure is the over-exuberant thickening of the vein graft wall beyond the level necessary for normal adaptation, known as neointimal hyperplasia

(NIH), without corresponding wall dilation, leading to decreased luminal size and stenosis. The molecular pathways contributing to this mechanism are complex. Endothelial denudation and disruption of endothelial signaling play an important role, while various other mediators such as oxidative stress and the inflammatory and coagulation cascades also contribute to wall thickening and failure. Eventually, these pathways result in modulation of extracellular matrix deposition and remodeling (**Figure 1**), mediated by transforming growth factor-β (TGF-β) and the matrix metalloproteinases (MMPs), respectively, and cell proliferation and migration, mediated by a variety of cytokines and growth factors such as VEGF, PDGF, bFGF, IGF, and other related molecules (3, 4, 8-14).

Over the years, our understanding of this failure mechanism has increased substantially, leading to a multitude of targeted experimental therapies to better control this process in order to improve the long-term outcomes of vein graft procedures (3, 4, 15, 16). However, these therapies have largely not been fruitful, as demonstrated by the disappointing results of the recent PREVENT III trials focusing on the E2F antagonist edifoligide (2, 15). As a result, there are still no successful methods to limit NIH, and the long-term failure rate of vein grafts remains high.

The Receptor Tyrosine Kinase Eph-B4

Recently, molecular distinctions between arteries and veins have been identified, including their differing genetic environments and embryologic determinations of identity (17). Especially important are the roles of Eph-B4 and its ligand Ephrin-B2, both part of the Eph (erythropoietin-producing hepatocellular carcinoma) family, the largest

family of receptor tyrosine kinases. Embryologically, Ephrin-B2 is a genetic determinant of arteries, while Eph-B4 is a genetic determinant of veins. However, while both are thought to remain markers of vascular identity, it is currently unknown whether they remain active in influencing the plasticity of arteries and veins even in adulthood.(1, 4, 18-21)

Our lab has sought to determine whether alterations in the expression of Eph-B4 and Ephrin-B2 may influence vein graft adaptation to the arterial environment. We have shown that vein graft adaptation in humans is characterized by loss of Eph-B4 expression without corresponding increase in Ephrin-B2 expression, signifying a loss of venous identity but without a corresponding gain in arterial identity (1). Furthermore, we have shown that increased expression of Eph-B4 may negatively inhibit neointimal hyperplasia, suggesting that Eph-B4 continues to play an important role in vessel plasticity and implicating Eph-B4 as an important molecular target for the pharmacological inhibition of vein graft failure due to neointimal hyperplasia (22).

Like all Eph receptors, Eph-B4 activation is strictly regulated, and occurs through auto-phosphorylation. Upon interacting with its ligand (Ephrin-B2) on the cellular surface, two inactive Eph-B4 kinase domains are brought together and dimerized. Immediately afterwards, the Eph-B4 dimer catalyzes the autophosphorylation of its own cytoplasmic domains, resulting in the commencement of its downstream effects. In addition to dimerization, clustering of many Eph-B4 domains leading to oligomerization may also occur (23).

However, the downstream mechanisms by which stimulation of Eph-B4 leads to decreased vein graft thickening is still unclear, and the elucidation of this mechanism

may open up new avenues of research and define novel ways to target this pathological mechanism of vein graft failure. Preliminary work in our lab has demonstrated that tyrosine 774 may be the tyrosine critical for Eph-B4 function, as mutations in Y774 lead to abolishment of Eph-B4's ability to phosphorylate the kinase Akt, one of the possible downstream effectors of Eph-B4 (24) (**Figure 2**). Finally, since Eph-B4 is upstream of the E2F pathway, modulation of Eph-B4 may provide significant therapeutic value, unlike the PREVENT trials.

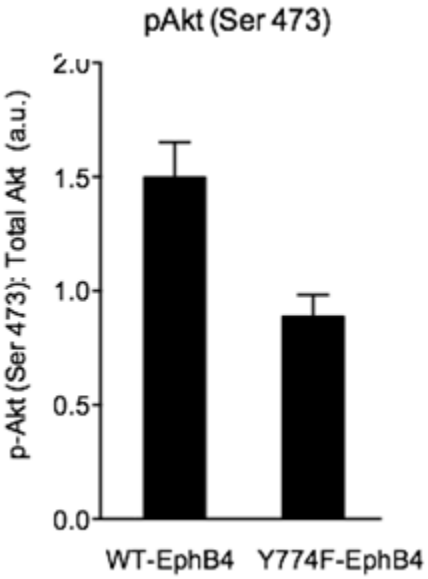


Figure 2. Preliminary data from our lab demonstrating loss of Eph-B4's ability to induce phosphorylation of Akt after stimulation with Ephrin-B2/Fc ($p = 0.0284$).

The Role of Extracellular Matrix in Vein Graft Adaptation

Although vascular smooth muscle cell proliferation and migration plays a large role in neointimal hyperplasia, coordinated extracellular matrix (ECM) deposition and remodeling is similarly critical. Removal and remodeling of existing extracellular matrix is necessary to allow venous dilation and cellular migration, while additional matrix deposition contributes to overall wall thickening (25). Westerband *et al.* demonstrated

that cellular proliferation and apoptosis in a vein graft is maximal in the first week after the procedure but is minimal afterwards, suggesting that any additional changes in wall thickness is likely due to extracellular matrix deposition (13). Additionally, continued increases in wall stiffness up to 6 months after vein graft implantation provides further evidence for the importance of matrix deposition during adaptation (26, 27).

Matrix deposition within vein graft walls is mediated by TGF-β, a cytokine produced from a variety of cell types, including inflammatory, endothelial, and smooth muscle cells (28). Upregulated expression of TGF-β and TGF-β receptor occurs soon after vein graft creation, while perioperative antisense inhibition of TGF-β expression leads to significant reduction in vein graft neointimal area (28, 29). Upregulation of TGF-β mRNA is further shown to be correlated with increased mRNA expression of both collagen I and collagen III (30). Jiang *et al.* demonstrated within rabbit vein grafts that progressive increases in neointimal mass up to 6 months after vein graft implantation is associated with minimal further cell proliferation, a decreased overall cell density, and a significantly increased proteoglycan content that occurs along with increased signaling of TGF-β and its downstream effector connective tissue growth factor (CTGF), again confirming the important contributions of extracellular matrix deposition to neointimal hyperplasia (25).

While TGF-β mediates ECM deposition, the matrix metalloproteinases regulate ECM remodeling, inducing the removal of collagen and elastin and possibly even the degradation of the internal elastic lamina to allow venous dilation and cell migration. The MMP family has been extensively studied and is stimulated by myriad factors that play a role during vein graft adaptation, including oxidative stress, inflammation, flow, stretch,

and even surgical injury itself (16, 31-33). Of these, MMP-2 and MMP-9 have been studied most extensively. Both demonstrate increased activity within the neointima of vein grafts soon after implantation but decline after 3-6 months, suggesting their importance to early vein wall remodeling and cell migration (25, 32, 34). Additionally, the surgical preparatory process itself, which includes adventitial stripping, ligation, and distension, has also been shown to increase MMP-2 and MMP-9 within the venous wall (35). A related molecule, MT1-MMP (MMP-14), an MMP-2 upstream activator, is also upregulated following vein graft implantation (34). Another related molecule, elastase, has also been shown to be upregulated, while elafin, an inhibitor of elastase, protects against neointimal hyperplasia in vein grafts (36). Meanwhile, TIMPs (Tissue Inhibitor of Metalloproteinase) are the main regulators of MMP activity, although their roles during vein graft adaptation is still unclear. TIMP-2 has been shown to be decreased early, followed by a return to baseline, while TIMP-1 has been shown to be upregulated (29, 34). Meanwhile, stimulation of TIMP-3 decreases neointimal hyperplasia (37).

The Role of eNOS in Vein Graft Adaptation

Surgical injury and irrigation, in addition to the new high/turbulent flow and high pressure hemodynamic environment, result in endothelial injury and denudation; denudation of the endothelium in a vessel may lead to exposure of the subendothelial collagen, loss of barrier function, and activation of the inflammatory and coagulation cascades (3, 9). Within vein grafts, endothelial denudation occurs within an hour after graft implantation, and the endothelial layer is not fully regenerated until 2 weeks after the procedure (8, 38). As the endothelium is thought to play a major role in regulating

both the caliber and adaptation of the vein, disruption of endothelial signaling in this manner potentially impairs the normal adaptive process, resulting in neointimal hyperplasia.

Among the vasoactive signals released by the vascular endothelium in responses to changes in its hemodynamic environment, nitric oxide (NO) has been the most extensively studied in the pathophysiology of vein graft adaptation. NO is created from endothelial nitric oxide synthase (eNOS), which is expressed by vascular endothelial cells, and is a potent vasodilator with additional anti-inflammatory, anti-thrombotic, and anti-proliferative properties (3, 4, 9). NO has been shown to limit macrophage infiltration, cell proliferation, and subsequent neointimal thickening within vein grafts (39, 40). Similarly, stimulation of eNOS and its related molecule inducible nitric oxide synthase (iNOS) may both inhibit neointimal hyperplasia, with iNOS additionally shown to inhibit adventitial thickening (40-42). Furthermore, surgical manipulation involves oxidative stress to the vein grafts during removal from the adventitial bed, disruption of the vasa vasorum, and subsequent ischemia-reperfusion. Superoxide, in particular, can deplete NO, leading to further disruption of its downstream effects (3, 43, 44). Finally and most importantly, preliminary experiments performed in our lab have suggested that Eph-B4 may play a significant role in eNOS signaling, as stimulation of Eph-B4 in mouse lung endothelial cells (MLECs) lead to two distinct peaks of eNOS phosphorylation (**Figure 3**).

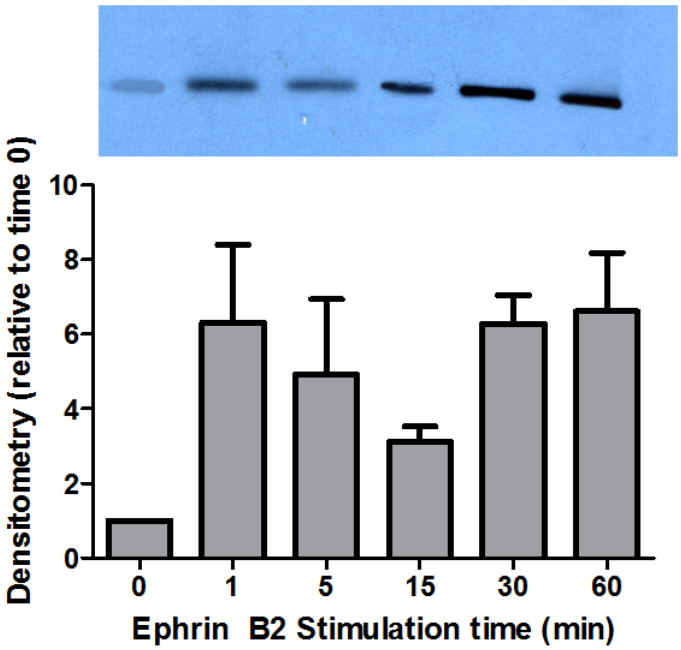


Figure 3. Preliminary data from our lab demonstrating Eph-B4 stimulation of eNOS phosphorylation within MLECs.

HYPOTHESIS AND SPECIFIC AIMS

Although we have demonstrated that stimulation of Eph-B4 reduces vein wall thickening during vein graft adaptation, the mechanism by which it accomplishes this function is still unclear. We believe that the elucidation of this mechanism will be integral in the search for new pharmaceutical targets based on Eph-B4 to manipulate vessel identity, control neointimal hyperplasia, and improve the long-term function of vein grafts in the future.

Given the importance of extracellular matrix deposition on vein graft adaptation, **we hypothesize that Eph-B4, directly or indirectly, reduces vein wall thickening at least partly through decreasing extracellular matrix deposition within the venous wall**. Furthermore, given that our preliminary data suggested that Eph-B4 may stimulate eNOS phosphorylation, **we hypothesize that Eph-B4 interacts directly with eNOS to stimulate its phosphorylation and mediate some of its downstream effects**.

Aim I: We will determine whether stimulation of Eph-B4 alters ECM deposition and remodeling. With *in vitro* cell culture experiments, we will stimulate cells with the ligand to the Eph-B4 receptor, Ephrin-B2, and quantify the mRNA and protein expression of ECM-related products compared to unstimulated cells. With *in vivo* mouse vein graft experiments, we will determine the effect of Eph-B4 stimulation or knockout on the ECM deposition within the walls of mouse vein grafts. Finally, in *ex vivo* experiments, we seek to determine the effect of Eph-B4 stimulation on collagen expression in human saphenous veins in culture.

Aim II: We will determine whether Eph-B4 directly interacts with eNOS to mediate some of its downstream effects. Given that our preliminary data suggested that Eph-B4 may mediate some of its effects either directly or indirectly through eNOS phosphorylation, we will determine whether Eph-B4 interacts directly with eNOS. We will transfect Eph-B4 and eNOS into a cell line that has no inherent expression of either Eph-B4 or eNOS and thus likely little of the cellular machinery associated with either. Any alterations of eNOS phosphorylation after Eph-B4 stimulation in this context will suggest a direct interaction between Eph-B4 and eNOS. Finally, if a direct interaction is established in this way, we will repeat this experiment with Eph-B4 Y774F to determine whether a mutation in tyrosine 774 abolishes Eph-B4's effect on eNOS, like it does for Akt.

MATERIALS AND METHODS

Antibodies and Reagents

Primary antibodies included: mouse Eph-B4 (R&D systems), mouse Ephrin-B2 (Abcam), mouse MMP2 (Cell Signaling Technology), mouse MMP9 (Abcam), HA-tag (Santa Cruz Biotechnology), total eNOS (Abcam), phospho-eNOS (Abcam), GAPDH (Cell Signaling Technology). Secondary antibodies included: HRP-conjugated anti-rabbit IgG (Cell Signaling Technology), HRP-conjugated anti-goat IgG (Cell Signaling Technology).

For stimulation experiments, mouse Ephrin-B2/Fc, IgG/Fc, and cd6/Fc chimeras (R&D Systems) were used for mouse cell culture and vein graft experiments, while human Ephrin-B2/Fc chimera (R&D Systems) was used for human vein culture experiments.

Cell Culture (MEF, HUVEC, MLEC, COS) and Eph-B4 Stimulation

MEFs (Mouse Embryonic Fibroblasts) and COS cells (monkey kidney fibroblast-like cell line) were cultured on high-glucose DMEM (Dulbecco's modified Eagle's medium) (Gibco Life Technologies) supplemented with 10% fetal bovine serum (FBS) (Gibco Life Technologies), L-glutamine, and penicillin/streptomycin. MLECs (Mouse Lung Endothelial Cells) and HUVECs (Human Umbilical Vein Endothelial Cells) were cultured in EBM-2 (Endothelial Basal Media-2) (Lonza) and supplemented with 15% FBS, L-glutamine, penicillin/streptomycin, and EGM-2 Bulletkit from Lonza, per manufacturer's recommendations. HUVECs used for experiments were at a maximum passage number of 6.

Prior to stimulation experiments, cells were plated on 6 well plates, grown to confluence, and starved in serum free media for 24 hours. Following starvation, cells were stimulated with either 2 ug/ml mouse Ephrin-B2/Fc or 2 ug/ml mouse IgG/Fc (negative control) (R&D Systems) in serum free media, for the designated amount of time, after which either the cell lysates or the conditioned media were collected.

RNA Extraction and qPCR (primers)

After completion of stimulation experiments, cells were lysed directly from their culture plates with Buffer RLT and RNA was extracted using the RNeasy kit (QIAGEN) and utilizing DNaseI for DNA digestion, per manufacturer's protocol. RNA concentration was measured with Nanodrop and RNA quality was checked through the 260/280 ratio, followed by normalization of RNA concentration. cDNA was then created through reverse transcription, utilizing SuperScript III First-Strand Synthesis Supermix (Invitrogen) per manufacturer's protocol. cDNA amplification utilized real-time, quantitative PCR with SYBR Green Supermix (Bio-Rad), with all samples normalized to GAPDH as the housekeeping gene. Melting curve analysis and gel electrophoresis was used to confirm primer efficiency and specificity, respectively.

mRNA was isolated from mouse muscle tissue from TRIzol reagent (Invitrogen), followed by extraction utilizing the RNeasy kit as described above. Mouse muscle tissue mRNA extraction and PCR were performed by Dr. Kota Yamamoto.

Primers utilized for mouse cell culture experiments were: Col1a1 (Collagen I): forward, 5'-CACCTGGTCCACAAGGTTTC-3', reverse, 5'-ACCATCCAAACCACTGAAGC-3'. Col3a1 (Collagen III): forward, 5'-

CAGGAGAAAATGGGAAACCA-3', reverse, 5'-GTCCAGCTCCACCTCTAGCA-3'. MMP2: forward, 5'-CAGGGCACCTCCTACAACAG-3', reverse, 5'-ACTTGTTGCCCAGGAAAGTG-3'. MMP9: forward, 5'-CATTCGCGTGGATAAGGAGT-3', reverse, 5'-ACCTGGTTCACCTCATGGTC. MMP14: forward, 5'-CCCTTTTACCAGTGGATGGA-3', reverse, 5'-TTTGGGCTTATCTGGGACAG-3'. Elastin: forward, 5'-GAGGTTTAGTGCCTGGTGGA-3', reverse, 5'-ATATGTCGGGATGCCAACTC-3'. TIMP1: forward, 5'-GCATCTGGCATCCTCTTGTT-3', reverse, 5'-CATTTCCCACAGCCTTGAAT-3'. TIMP2: forward, 5'-AAGCAGTGAGCGAGAAGGAG-3', reverse, 5'-TTCTTTCCTCCAACGTCCAG-3'. GAPDH: forward, 5'-AATGTGTCCGTCGTGGATCTGA-3', reverse, 5'-AGTGTAGCCCAAGATGCCCTTC-3'.

Primers utilized for human vein graft culture experiments were: Col1a1: forward, 5'-CTGGTCCTGATGGCAAAACT-3', reverse, 5'-AGCTCCAGCCTCTCCATCTT-3'. GAPDH: forward, 5'-GAGAAGGCTGGGGCTCATTT-3', reverse, 5'-AGTGATGGCATGGACTGTGG-3'.

Human Saphenous Vein Culture

Remnants of excess saphenous vein (n=7) from coronary bypass procedures were procured, per HIC protocol #9908011041, cut into cross-sectional rings, and placed into culture (RPMI 1640, Gibco, with 30% FBS, 1% L-glut, pen/strep) at 37°. Each vein was divided into 3 segments. After blinding, one segment from each vein was cultured but unstimulated for 14 days, one segment was stimulated with 2ug/ml Ephrin-B2/Fc (R&D

Systems), and one segment was immediately processed as Day 0 without culturing. Samples were the frozen in optimal cutting temperature compound (OCT, Tissue-Tekl Sakura Finetek), followed by homogenization and RNA purification using RNeasy Mini Kit (Qiagen), per manufacturer's protocol, and finally qPCR with primers as described previously. All human saphenous vein culture steps prior to qPCR were performed by fellow lab member Daniel Wong, as detailed in his own thesis dissertation. All qPCR data for each sample (stimulated, unstimulated) was normalized to GAPDH as a housekeeping gene, and then normalized to the corresponding Day 0 sample.

Quantification of Collagen-related Protein expression

For Western Blots, cell lysates were collected from the cell culture plates with RIPA buffer after the specified stimulation times, followed by sonication of each sample for disruption of cell membranes. Protein concentrations were measured utilizing BioRad Protein Assay and normalized. Equal amounts of protein from each sample were loaded onto 10% SDS-PAGE gels, followed by transfer for Western blot. The membranes were then incubated with the specified antibodies, overnight for primary, and 1 hour for secondary, as described under Antibodies and reagents, followed by film exposure and development. Quantitative analysis of western blot bands was performed by densitometry with ImageJ. Cell counts, when necessary, were performed with a haemocytometer and Trypan Blue.

For quantification of total collagen expression, we utilized the Sircol Collagen Assay (Biocolor Life Science Assays). For each n, 3-7 replicates were used. Briefly, cells were stimulated as above, except stimulation occurred immediately when cultured to

account for collagen deposition in first 24 hours. Afterwards, microwell plates were subject to 24 hours of collagen extraction in 0.1mg/ml of pepsin (Sigma) in 0.5 M acetic acid at 4º, followed by isolation of collagen with the Sircol Assay, per manufacturer's protocol. Final collagen quantification was performed with a microwell plate reader at a wavelength of 550nm, corresponding to the peak absorption of the Sirius Red-based dye. Each measurement n (total n=6) was the mean of 3-7 replicates. As the same concentration of cells was used in all the replicates of a given n, measurements for both control and stimulated groups were normalized to that of the control group.

Gelatin Zymography

After the specified stimulation times, conditioned media from the cell culture plates were collected and added to equal volumes of Laemmli Sample Buffer (BioRad). The resulting mixtures were then ran through 10% SDS-PAGE gels with 10% porcine skin gelatin (Sigma). Gels were then renatured with renaturing buffer (2.5% Triton-X in water v/v) for 30 minutes, followed by incubation at 37° for 2-4 hours in developing buffer (per Millipore Zymography protocol). Finally, gels were stained with .25% Coomassie Blue-R250, followed by destaining with destaining solution (per Millipore Zymography protocol). Quantitative analysis of zymography bands was performed by densitometry with ImageJ.

Histological Quantification of Collagen in Mouse Vein Grafts

Mouse vein graft sections were obtained from prior lab members, per their previous surgical and experimental procedure.(22) Briefly, the intrathoracic inferior vena

cava (IVC) was excised and interposed into the infrarenal abdominal aorta in C57BL/6 mice. Experimental groups included wild type IVC transposed into wild type aorta (WT WT), wild type IVC transposed into Eph-B4 knockout aorta (WT KO), Eph-B4 knockout IVC transposed into wild type aorta (KO WT), and Eph-B4 knockout IVC transposed into Eph-B4 knockout aorta (KO KO). Other experimental groups included wild type IVC transposed into wild type aorta along with systemic stimulation every 48 hours with either Ephrin-B2/Fc or control cd6/Fc. 4 weeks after the procedure, vein grafts were excised and sectioned. Vein graft sections were obtained from said lab member and Masson's Trichrome stain was performed on all sections by the pathology lab staff at Yale School of Medicine. Quantification of collagen (blue) within the vascular wall as well as lumen circumference were done utilizing Photoshop and ImageJ software, after blinding by a fellow lab member. Total area of blue within the venous wall was divided by inner luminal circumference in order to determine average thickness of collagen in each vein graft wall.

Cell Transfection and Eph-B4 Stimulation

COS cells were plated in antibiotic-free growth medium (DMEM, 10% FBS, L-glut) and grown to confluence. Media in the cell plate was then removed and replaced with 1ug of specified plasmids was mixed with 2ul of Lipofectamine 2000 (Life Technologies) in OptiMEM media (Life Technologies), per manufacturer's protocol. For double transfections, 1ug of each plasmid was added. Transfected plasmids included eNOS, hemagglutinin-tagged Eph-B4, hemagglutinin-tagged Eph-B4 774 mutant (E-774), pcDNA13 (control vector plasmid for eNOS), p-shuttle (control vector plasmid for

Eph-B4 and E-774). Cells were then incubated for 6 hours in a 37º CO_2 incubator, followed by replacement of media with serum-free, antibiotic-free medium (DMEM, L-glut). After continued incubation at 37º for 24 hours, stimulation experiments with Ephrin-B2/Fc or control cd6/Fc were performed as previously described, followed by extraction of cell lysates in RIPA buffer containing phosphatase-inhibitors NaF and Sodium Orthovanadate. Protein quantification and western blotting was then performed as previously described, with anti-HA (hemagglutinin) used to detect Eph-B4 due to antibody quality. After densitometric measurement of band size, ratio of phospho-eNOS to total-eNOS was calculated in order to quantify status of eNOS phosphorylation within samples.

Statistical Analysis

For all experiments for which $n \geq 4$, statistical analysis was performed by comparison of treatment groups with control group utilizing a 1 sample t-test or an independent, 2-sample t-test, with $p < 0.05$ considered significant. All bar graphs represent sample means with error bars representing standard errors of the means in either direction.

RESULTS

Selection of Cells for ECM Experiments

To select cells for use for cell culture experiments, we performed measurements of mRNA and protein expression of Eph-B4 at baseline in various cell types. For experiments on extracellular matrix deposition, an immortalized fibroblast cell line was desired, given that ECM deposition is a known function of fibroblasts, while the involvement of endothelial cells on ECM deposition is unclear. In order to determine the role of Eph-B4 on ECM deposition, a cell line that expresses Eph-B4 at baseline was required. Mouse embryonic fibroblasts (MEFs) express both Eph-B4 protein and mRNA at baseline, compared to positive controls (MLECs) and negative controls (COS cells, mouse muscle tissue) (**Figure 4A, C**). MEF expression of Eph-B4 normalized to GAPDH was 35-fold higher than negative control mouse muscle tissue, although it was not as high as MLEC expression of Eph-B4 (286-fold higher than mouse muscle tissue) (**Figure 4C**). Protein expression of Eph-B4 paralleled this trend (**Figure 4A**). Thus, MEFs were selected for all ECM experiments. Of note, all cell types studied expressed Ephrin-B2 at baseline (**Figure 4B**).

Eph-B4 has no effect on mRNA expression of various ECM-related products

To examine the effect of Eph-B4 stimulation on ECM deposition and remodeling, MEFs were stimulated with Ephrin-B2/Fc or control IgG/Fc for various durations, followed by quantification of mRNA expression of various ECM-related products. Stimulation of Eph-B4 in MEFs with Ephrin-B2/Fc or IgG/Fc had no effect on mRNA

expression of Collagen I, Collagen III, MMP2, and MMP9 after 4 hours or 12 hours (n=2 for each group) (**Figure 5A**). Similarly, stimulation of Eph-B4 in MEFs had no effect on mRNA expression of MMP14, elastin, TIMP1 and TIMP2 after 4 hours or 24 hours (n=3 for each group) (**Figure 5B**).

In order to study changes in extracellular matrix production which may occur over a longer time course of days, we further extended the period of stimulation to up to 5 days in culture. Compared to control cells at the beginning of the stimulation period (n=1), all cell culture groups – Unstimulated, Ephrin-B2/Fc, Cd6/Fc (n=2 for each group) – displayed increased mRNA expression of collagen I, collagen III, MMP2, and MMP9 that peaked for each product by the 3rd day of stimulation. This increase was maintained up to the 5th day for MMP2, but returned to baseline for all other products (**Figure 5C**). Most notably, there was no difference in the pattern of mRNA expression at any point during the 5 day duration between the Ephrin-B2/Fc stimulated samples and the samples in the two control groups, for all 4 products.

Finally, we examined the effect of Ephrin-B2/Fc stimulation on collagen expression of *ex vivo* human saphenous vein grafts in culture. There was no change in the mRNA expression of Collagen I in the Ephrin-B2/Fc stimulated group after 21 days of stimulation compared to either the unstimulated group (p=0.948) or the Day 0 uncultured group (p=0.834) (n=7 for each group) (**Figure 5D**).

Eph-B4 has no effect on protein expression or activity of various ECM-related products

To examine total collagen deposition, we measured total collagen deposited onto cell plate walls by MEFs. There was no significant difference in total collagen deposited after 7-8 days, utilizing the Sircol Collagen Assay (n=6) (p=0.127) (**Figure 6A**). There was also no difference in total MMP2 and MMP9 intracellular protein expression between stimulated and unstimulated cells, as measured by Western Blot of the cell lysates (n=2-3) (**Figure 6B**) after both 6 and 48 hours, although total MMP2 and MMP9 did increase with additional time of culture, corresponding to their mRNA levels, as described above. Negligible elastin protein expression was identified in all samples (data not shown).

We then examined the total activity of MMP2 intracellularly and within conditioned media by zymography. There was no change in total MMP2 activity within the conditioned media of the cells after 6 hours of stimulation (which accounts for secretion of MMP2 by cells and degree of activation/post translational modification) (p=.481, n=3-4) (**Figure 7A-B**). The results were similar with normalization of zymography densitometry to either total protein in each sample or to approximate cell number within each sample (data not shown). Likewise, there was no change in total MMP2 activity within the cell lysates after either 6 hours or 48 hours of stimulation (which accounts for protein expression by cells and degree of activation/post-translational modification) (**Figure 7C**).

Eph-B4 has no effect on overall collagen deposition in mouse vein grafts

To examine the effect of Eph-B4 on vein grafts, veins from wild type (WT) or Eph-B4 knockout (KO) mice were grafted into the aortas of wild type or Eph-B4

knockout mice. After 4 weeks, vein grafts were sectioned and stained with Masson's Trichrome to quantify total collagen present within vein graft walls. Blue-staining collagen was mostly apparent in the adventitial layer, with the combined mouse intima-media layer containing both red and blue (**Figure 8A**). There was no difference in average thickness of the collagen blue in the vein graft walls among any of the four knockout groups compared to control WT WT – WT KO (p=0.697), KO WT (p=0.937), KO KO (p=0.215) (n=4-5 for each group) (**Figure 8B**). There was also no significant difference in average lumen circumference of the vein graft between any of the 3 groups compared to control WT WT (p>0.088 for all 3 groups) (n=4 per group) (**Figure 8B**).

To confirm these results, a similar experiment was performed in which wild type vein grafts were interposed into the aortas of wild type mice (WT WT), but with half the group undergoing stimulation with systemic Ephrin-B2/Fc or control Cd6/Fc. In this case, there was no difference in average thickness of the collagen blue in the vein graft wall (p=0.808) and no difference in average lumen circumference (p=0.636) between the two groups (n=3-4 per group) (**Figure 8C**).

Eph-B4 interacts directly with eNOS to produce eNOS phosphorylation

Since Eph-B4 does not appear to regulate vein graft adaptation by regulation of ECM volume or components, we next examined whether regulation of eNOS would be a potential mechanism of Eph-B4 action. For experiments on the interaction of Eph-B4 and eNOS, a cell line that exhibits high transfection efficiency yet does not express Eph-B4 and eNOS at baseline is required. COS cells do not express Eph-B4 or eNOS protein at

baseline (**Figure 4A**, **Figure 9A**, **Figure 10A**), and do not express Eph-B4 mRNA at baseline (from prior lab members, data not shown).

COS cells were transfected with either Eph-B4, eNOS, both, or the combination of the vector plasmids (P-S and pcDNA). Confirmation of transfection is shown in **Figure 9A**, where only cells with transfected plasmid expressed that product on Western Blot. The results strongly suggest a direct interaction between Eph-B4 and eNOS. The proportion of phosphorylated transfected eNOS is massively increased simply by the simultaneous transfection of Eph-B4, without requiring Eph-B4 stimulation. Stimulation of Eph-B4 with Ephrin-B2/Fc then increased this proportion even further. As expected, stimulation with Ephrin-B2/Fc in the absence of simultaneous Eph-B4 transfection did not increase the proportion of phosphorylated eNOS (**Figure 9B**). A dual peak of eNOS phosphorylation is apparent with a rapid early peak after 1-5 mins of stimulation and a later peak beginning at 30 mins of stimulation (**Figure 9C**). This bimodal distribution exactly matches that seen in our preliminary data within MLECs (data by prior lab members, data not shown).

Tyrosine 774 affects but is not critical to Eph-B4's interaction with eNOS

Given that a mutation in Tyrosine 774 abolishes Eph-B4's effect on Akt phosphorylation, we repeated the transfection experiments utilizing a mutation in Eph-B4 at tyrosine 774 (E-774). As previously, confirmation of transfection is shown in **Figure 10A**. E774 did not abolish Eph-B4's ability to phosphorylate eNOS, as the proportion of phosphorylated transfected eNOS is increased, again, simply by the simultaneous transfection of E774 with eNOS, followed by a further increase upon stimulation of E774

with Ephrin-B2/Fc (**Figure 10B**). Similarly, the dual-peaked temporal pattern of eNOS phosphorylation upon Eph-B4 stimulation is preserved (**Figure 10C**).

However, although increased, these amount of these increases is substantially less than the increases observed with transfection and stimulation of wild-type Eph-B4. Simultaneous transfection with either WT Eph-B4 or E-774 both led to an approximately 3.8-fold increase in proportion of phosphorylated transfected eNOS. However, further stimulation with Ephrin-B2/Fc led to a smaller increase in E774 (4.4-fold) compared to that in wild type Eph-B4 (**Figures 9B, 10B**). Similarly, although the dual-peaked temporal pattern is preserved, the peaks are diminished and delayed. The early peak, which began as early as after 1 minute of stimulation with a max increase in phosphorylated eNOS of 6.6-fold, is delayed, only appearing after 5 minutes of stimulation and with a max increase of only 4.4-fold. Similarly, the later peak, which began as early as after 30 minutes of stimulation with a maximum of 5.9-fold increase in phosphorylated eNOS in the WT Eph-B4 transfections, is delayed to sometime after 30 minutes, with a maximum measured increase of 4.7-fold in E774 (**Figures 9C, 10C**). Thus, unlike Eph-B4's relationship with Akt, the presence of tyrosine 774 affects Eph-B4's effects on eNOS phosphorylation but does not appear to be absolutely critical.

DISCUSSION

Vein graft failure due to neointimal hyperplasia continues to be a significant source of patient morbidity and mortality, while being a significant siphon to healthcare expenditure in the United States. Thus far, advances in knowledge of vascular biology has led to many prospective therapies to fix this problem, but thus far none have successfully translated clinically, and we are continually looking for new ways to control the optimal amount of vein wall thickening necessary for proper adaptation to the arterial environment without the stenosis that occurs with pathological, over-exuberant wall thickening.

Vein wall thickening requires both cell proliferation and extracellular matrix deposition (13, 26, 27). The receptor tyrosine kinase Eph-B4, an embryologic determinant of venous identity, is not only a marker of venous identity in adulthood but appears to continue to influence vascular plasticity, as vein grafts lose their venous identity but does not gain an arterial identity as part of the adaptation process to the arterial environment, while stimulation of Eph-B4 results in decreased vein graft wall thickening (1, 22). However, we do not currently know the mechanism by which Eph-B4 mediates any of its downstream effects. Given the importance of extracellular matrix deposition and remodeling to vein graft adaptation, we sought to determine whether Eph-B4 accomplishes its effect of by decreasing extracellular matrix deposition and remodeling, in order to better understand how to it can be utilized as a potential therapeutic target in clinical trials in the future for the pharmacological control of vein graft stenosis and failure.

Through a series of *in vitro*, *in vivo*, and *ex vivo* experiments, we have shown that Eph-B4 has little effect on ECM deposition and remodeling. Stimulation of Eph-B4 led to no changes in mRNA expression of Collagen I, Collagen III and elastin, at both early and late timepoints. Of note, Collagen I and Collagen III were selected among the myriad collagens as they are the collagens most prevalent in the vascular system and most responsible for both tensile strength and elasticity (45). Similarly, stimulation of Eph-B4 led to no changes in mRNA expression of MMP2 and MMP9, the two matrix metalloproteinases most often implicated in vascular remodeling, as well as TIMP1 and TIMP2, the major regulators of MMP activity. *Ex vivo*, human saphenous veins displayed no alterations in collagen mRNA expression following 14 days of Eph-B4 stimulation in culture. Paralleling the lack of effect on collagen-related mRNA products, Eph-B4 stimulation also had no effect on cellular protein expression of MMP2 and MMP9, no effect on total collagen secreted by cells, and no effect on total secreted and intracellular MMP2 activity as measured by gelatin zymography, where total activity additionally accounts for protein expression, active cellular secretion, and post-translational activation of MMP2.

The study of extracellular matrix is vast, and this study is limited in the aspects of ECM deposition and remodeling that can be studied. For example, there are at least 19 collagens identified, while 13 of them can be found in the vascular wall (45). Many other related molecules, such as elastin and proteoglycans, may also play a significant role in remodeling the caliber and elasticity of the vascular wall. Additionally, the degradation of collagen not only involves more than 24 classes of matrix metalloproteinases, but also other related molecules such as a variety of cathepsins and elastases (46, 47). There are

also at least four known mammalian TIMPs (48). Finally, as previously mentioned, other related signaling pathways, like TGF-β, also play a very significant role, and the interaction between all of these components are complex. We focused our experiments on only the components that were most prominent within vascular remodeling (Collagen I, Collagen III, elastin, MMP2, MMP9, MMP14, TIMP1, TIMP2), and a completely thorough examination of Eph-B4 expression on all aspects of ECM deposition/remodeling would be difficult. Although it is possible that Eph-B4 may affect the expression and activity of some of these other components, our *in vivo* mouse studies, in which Eph-B4 stimulation resulted in no significant histological difference in overall collagen deposition into the walls of the vein graft, suggests that this is unlikely.

Another limitation of the extracellular matrix experiments is that a majority of the molecular studies were performed on cells or veins in culture, which may not be representative of a vein graft *in vivo*. Furthermore, the experiments utilized only a few, selected time points thought to be representative, while true changes in mRNA and protein expression and activity may occur at any number of time points that were not measured. However, again, despite these limitations, our *in vivo* results showing no overall change in collagen deposition 4 weeks after vein graft implantation provides confirmation of our molecular studies within culture.

Inspection of the vein graft sections after Trichrome staining revealed that the majority of blue-staining collagen seemed to be present within the adventitia, and only some collagen present in the intima-media layer. Combined with previous studies suggesting that it is the thickness of the neointimal layer that is affected by Eph-B4 (22), these results suggest that Eph-B4 likely reduces vein wall thickening not through

reducing extracellular matrix deposition, but mainly through decreasing smooth muscle cell proliferation and migration within the intima, which should be the main focus of future studies on the mechanism of Eph-B4.

Finally, we were able to demonstrate a direct interaction between Eph-B4 and eNOS. Although our preliminary data had shown that Eph-B4 induced a dual-peak of phosphorylation of eNOS within MLECs, it was not determined whether this effect was a direct interaction between the two or whether this was an indirect effect. We were able to demonstrate a very similar dual-peaked temporal profile of eNOS phosphorylation after Eph-B4 stimulation within transfected COS cells. Furthermore, we were able to show that transfection of Eph-B4, even without further stimulation, is enough to greatly increase the amount of eNOS phosphorylation. By demonstrating this data in a transfected cell line that has no inherent expression of Eph-B4 and eNOS and thus likely has little of the associated cellular machinery associated with the two, these results strongly suggest a direct interaction between Eph-B4 and eNOS within the cell, with eNOS likely directly mediating many of Eph-B4's downstream effects.

Furthermore, we replicated the double transfection experiments with a version of Eph-B4 containing a mutation of Tyrosine 774 (Tyrosine → Phenylalanine). In our search for the critical tyrosines that mediates either Eph-B4 auto-phosphorylation or its downstream phosphorylation functions, tyrosine 774 has been previously implicated by our lab as one such critical tyrosine. Although we have shown that this exact Y774F mutation leads to abolishment of the phosphorylation of Akt, one of the downstream effectors of Eph-B4, it was unknown whether this mutation led to an isolated decrease in the phosphorylation of Akt by Eph-B4, or whether the mutation leads to a complete

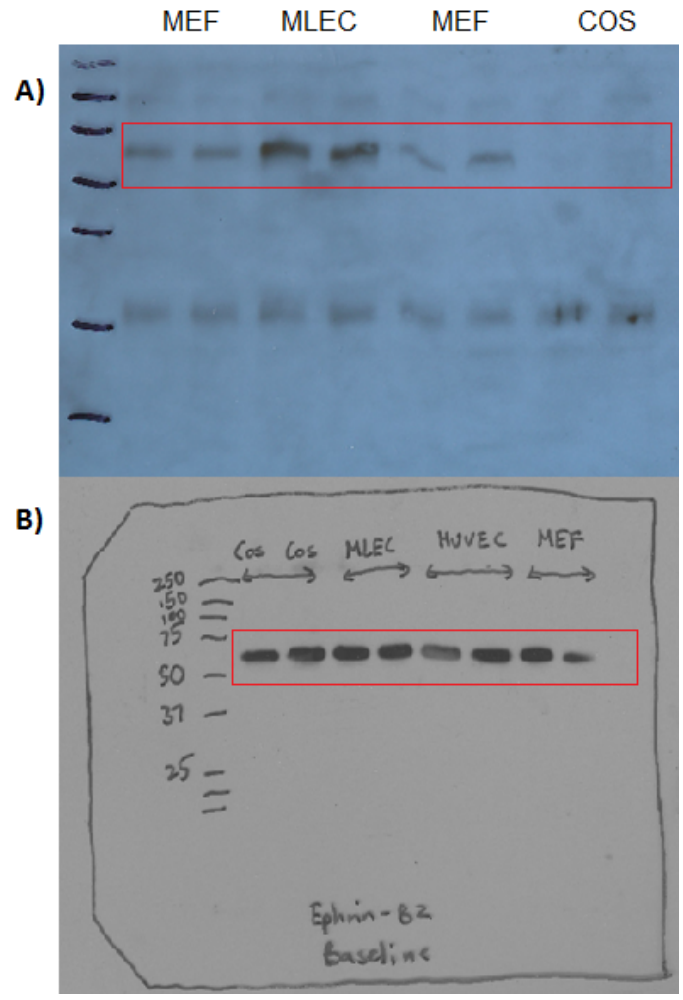
destruction of all Eph-B4 activity, either through inducing a complete protein misfolding or through destroying the Eph-B4 receptor's ability to auto-phosphorylate and activate itself upon encountering its ligand.

By demonstrating that the Y774F mutation preserves the effect of Eph-B4 on eNOS phosphorylation, our results suggest that tyrosine 774 is not crucial to all Eph-B4 downstream function, is not required for Eph-B4 self-activation, and that Y774F does not induce a global protein misfolding. These results also suggest that tyrosine 774 is likely the crucial tyrosine specifically for Eph-B4's interaction with Akt. Although we did observe a minor diminishment of total eNOS phosphorylation and a minor delay in the two peaks of phosphorylation, the most likely explanation is that although tyrosine 774 is not crucial to Eph-B4's interaction with eNOS, the mutation leads to minor conformational changes that result in a slightly decreased efficiency of the Eph-B4 receptor. Future studies will look to further examine the other remaining tyrosines to determine the crucial tyrosine(s) responsible for the interaction of Eph-B4 and eNOS, as well as the tyrosine(s) responsible for Eph-B4 auto-phosphorylation and activation. Several other tyrosines have already been established by our lab as potentially important targets, such as tyrosines 821 and 924. As eNOS may mediate a large variety of important vascular functions, future studies will also examine which of the downstream effects of Eph-B4 is mediated specifically by eNOS. Since Eph-B4 does not affect ECM deposition but instead targets eNOS, future *in vitro* molecular studies on Eph-B4 should be performed on endothelial cells (mediators of signaling and from which eNOS is secreted) rather than the fibroblasts used in these experiments.

In conclusion, Eph-B4 stimulation has little effect on extracellular matrix deposition, specifically not on the expression and activity of collagen I, collagen III, MMP2, MMP9, MMP14, elastin, TIMP1, TIMP2, and not on overall collagen deposition by fibroblast cells *in vitro* and mouse vein graft walls *in vivo*. Eph-B4's effects on decreasing vein wall thickening likely occurs through modulation of vascular smooth muscle proliferation and migration instead. Furthermore, Eph-B4 likely interacts directly with eNOS to mediate some of its downstream effects, and tyrosine 774, although crucial to the interaction of Eph-B4 with Akt, is not crucial for global function of Eph-B4 or the interaction of Eph-B4 with eNOS.

FIGURES AND LEGENDS

Figure 4. Baseline expression of A) Eph-B4 (~120 kDA) and B) Ephrin-B2 (~55 kDA) in multiple cell types. C) mRNA expression of Eph-B4. MEF = Mouse Embryonic Fibroblasts, MLEC = Mouse Lung Endothelial Cells, COS = cos-7 cells/Monkey kidney fibroblast cells, HUVEC = Human Umbilical Vein Endothelial Cells.



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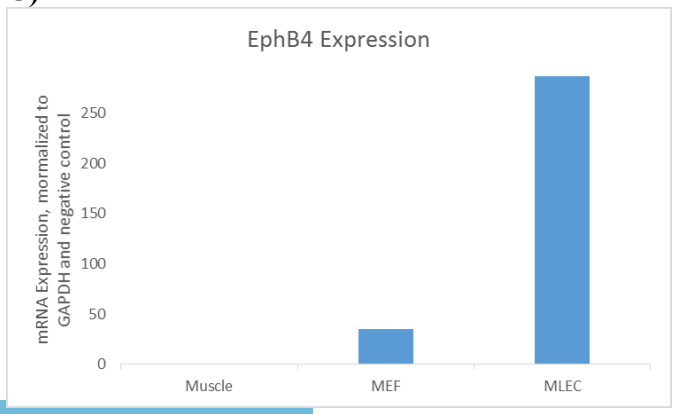

Figure 5. mRNA Expression of Collagen-related products after stimulation of Eph-B4. A) Expression of collagen-related products in MEFs after 4 hours (upper panel) or 12 hours (lower panel) of stimulation with control IgG/Fc or Ephrin-B2/Fc, normalized to the unstimulated expression, n=2. **B)** Expression of additional products after 4 hours (upper panel) or 24 hours (lower panel). **C)** Expression of collagen-related products in MEFs after up to 5 days of stimulation with control IgG/Fc or Ephrin-B2/Fc (n=2), normalized to MEF isolated at the beginning of the experiment (n=1). **D)** Expression of collagen I in untreated or Ephrin-B2/Fc treated human veins in culture after 14 days, with each sample normalized to corresponding uncultured Day 0 human vein (n=7 for each group). Col1a1 = Type I Collagen, Col3a1 = Type III Collagen, MMP = Matrix Metalloproteinase. TIMP = Tissue Inhibitor of Metalloproteinase.

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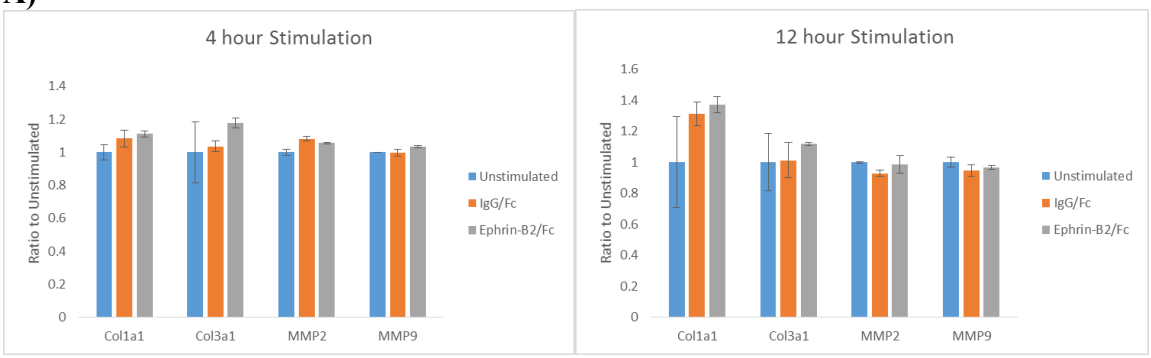

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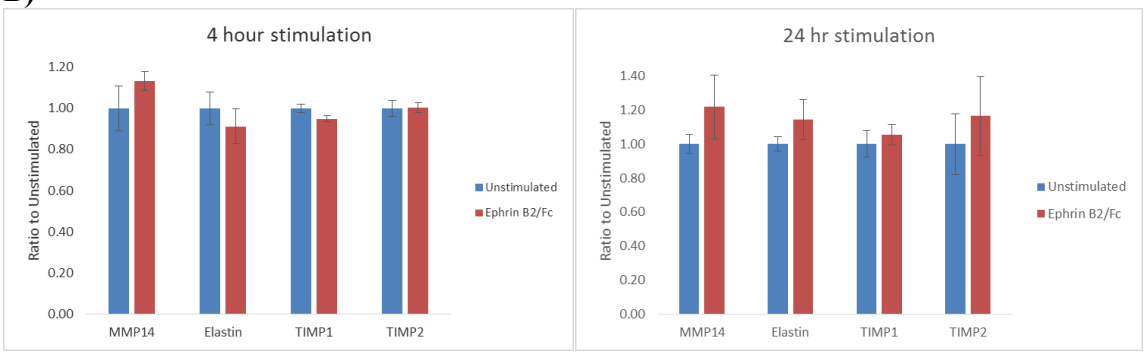

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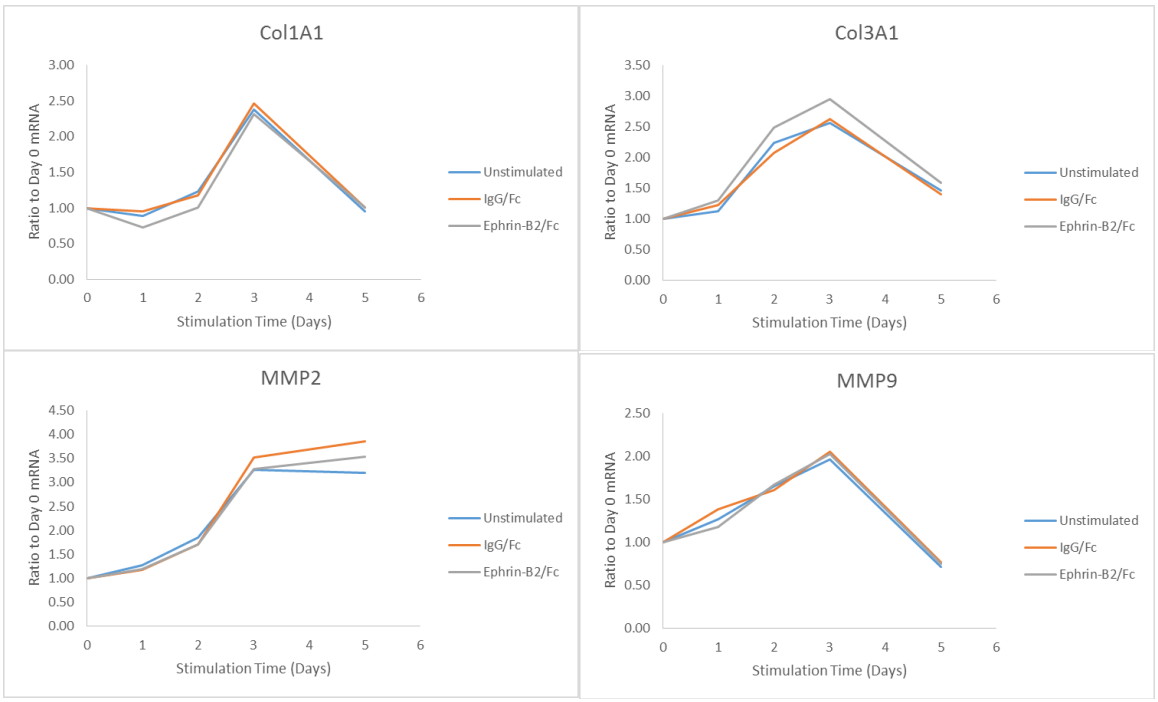

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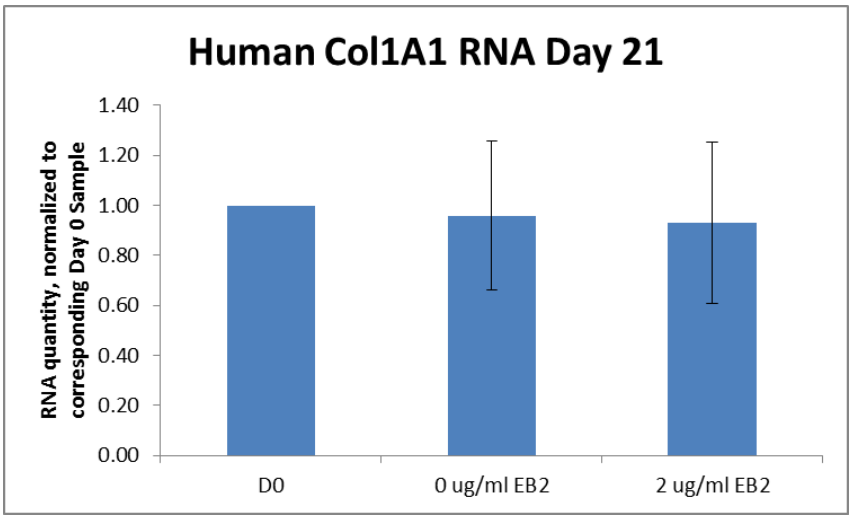

Figure 6. Protein expression of collagen-related products after stimulation of MEFs in culture. A) Total collagen secreted onto the cell culture plate (n=6), normalized to control group. **B)** Western Blot of MMP2 and MMP9 within MEF cell lysates after 6 hours or 48 hours of stimulation. Cd6 = cd6/Fc. EB2 = Ephrin-B2/Fc. Unstim = Unstimulated.

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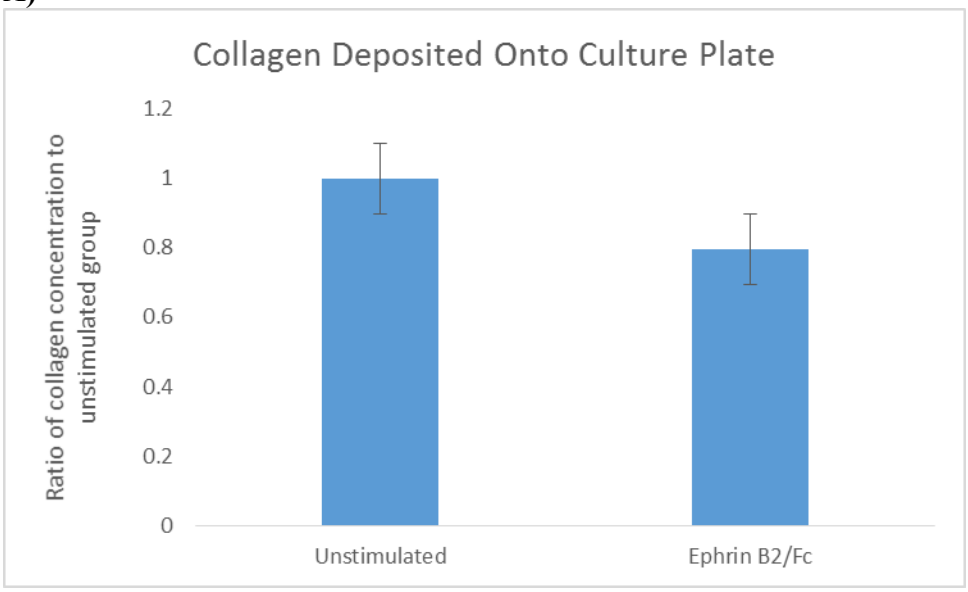

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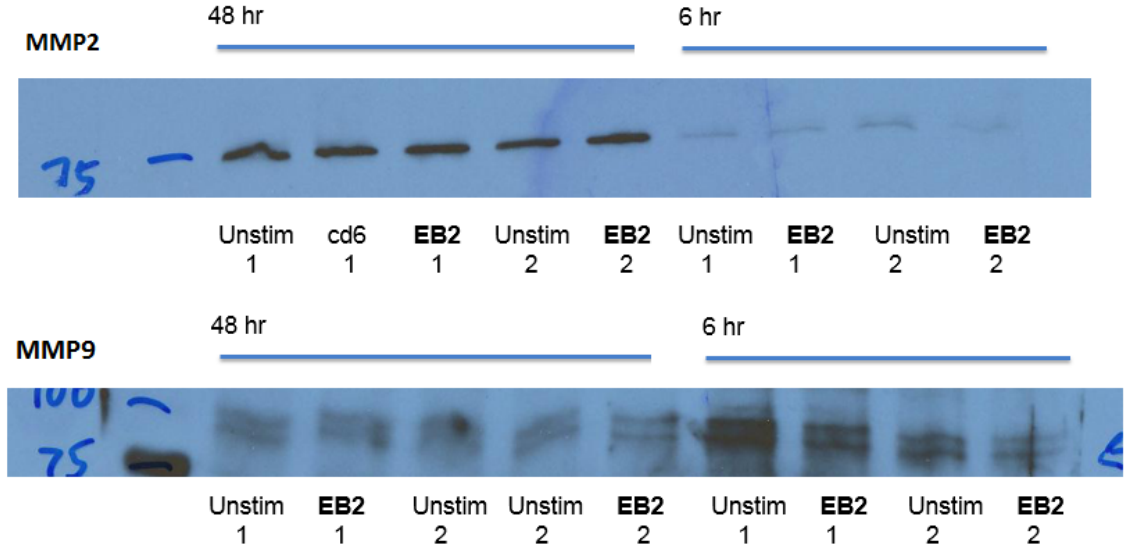

Figure 7. Activity of MMP-2 and MMP-9 measured by zymography after stimulation of Eph-B4 with Ephrin-B2/Fc in MEFs. A) Zymography of MMP-2 activity in conditioned media of (secreted by) unstimulated (U, n=4) and stimulated (E, n=3) cells. **B)** Densitometry comparisons of bands representing MMP-2 activity in conditioned media (secreted by) stimulated and unstimulated cells. **C)** Densitometry comparisons of bands within cell lysates of stimulated and unstimulated cells (n=2 for each group).

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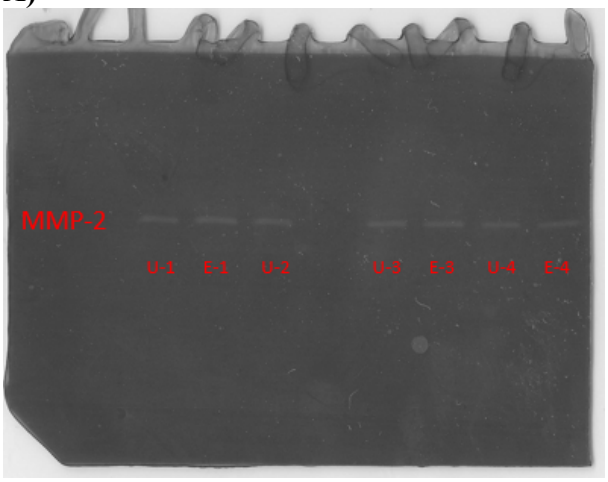

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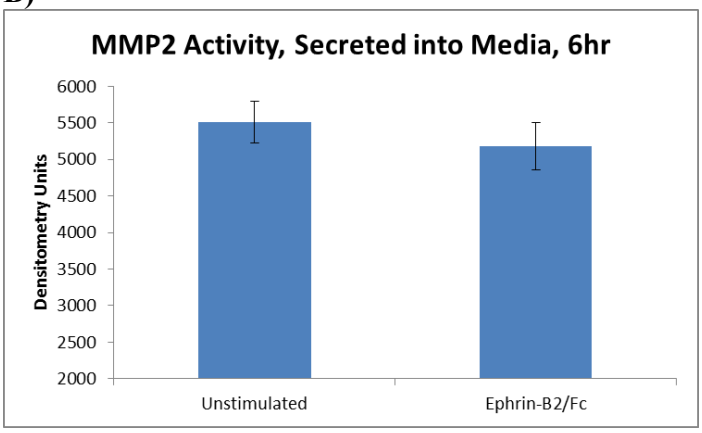

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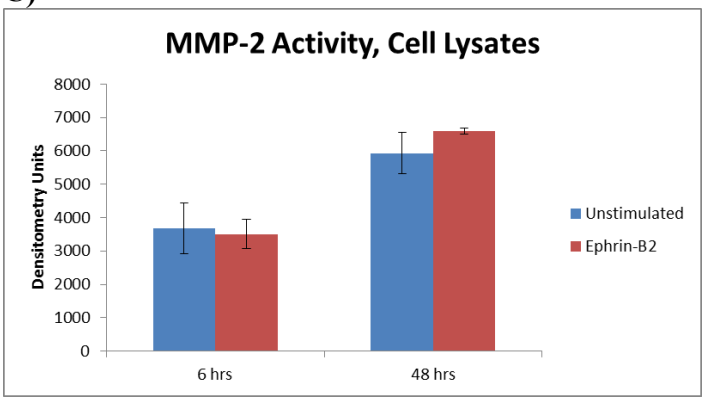
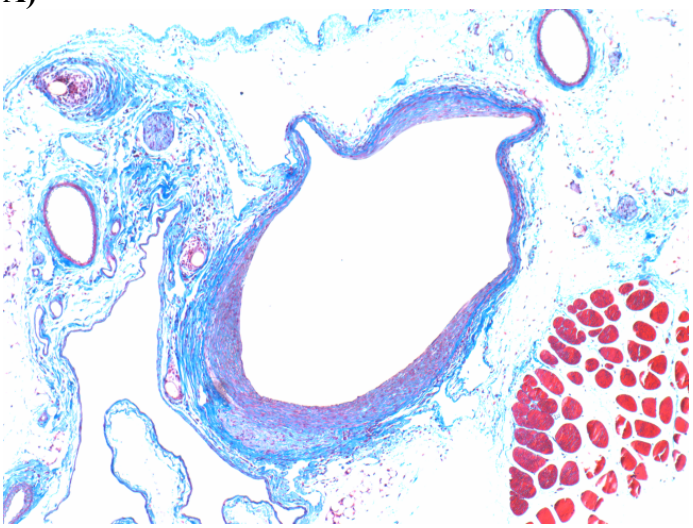


Figure 8. Histological quantification of total collagen and lumen circumference within Masson's Trichrome stains of sections of vein grafts in mice. **A)** Sample Trichrome stain of vein graft section. **B)** Average wall thickness and lumen circumference of 4 week old vein grafts in Eph-B4 knockout experiment. **C)** Average wall thickness and lumen circumference of 4 week old vein grafts in Eph-B4 stimulation experiment. WT = Wild type. KO = Knockout. EB2 = Ephrin-B2/Fc. Cd6 = cd6/Fc.

A)



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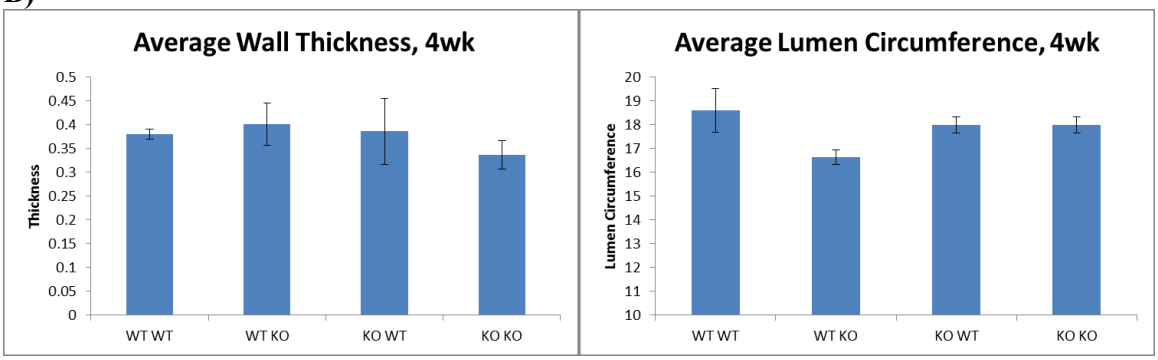

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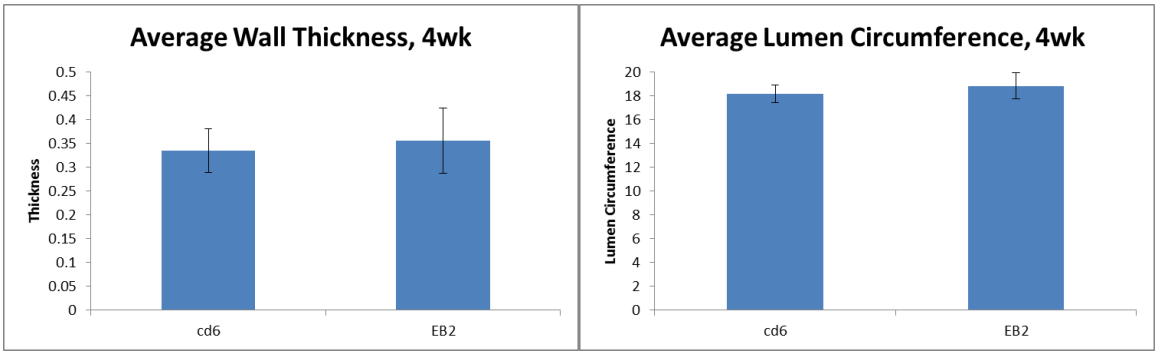

Figure 9. Eph-B4 and eNOS transfection within COS cells. A) Eph-B4, eNOS, or both were transfected into COS cells, stimulated with designated ligand, and cell lysates were taken for Western blot to measure Eph-B4, total eNOS, or phospo-eNOS. **B)** Densitometry quantification of ratio of p-eNOS to total eNOS. **C)** Densitometry quantification of ratio of p-eNOS to total eNOS, normalized to ratio in unstimlated samples. PS = P-Shuttle (vector control for Eph-B4 plasmid). pcDNA = pcDNA13 (vector control for eNOS plasmid). EB2 = Ephrin-B2/Fc. Cd6 = cd6/Fc (control for Ephrin-B2/Fc).

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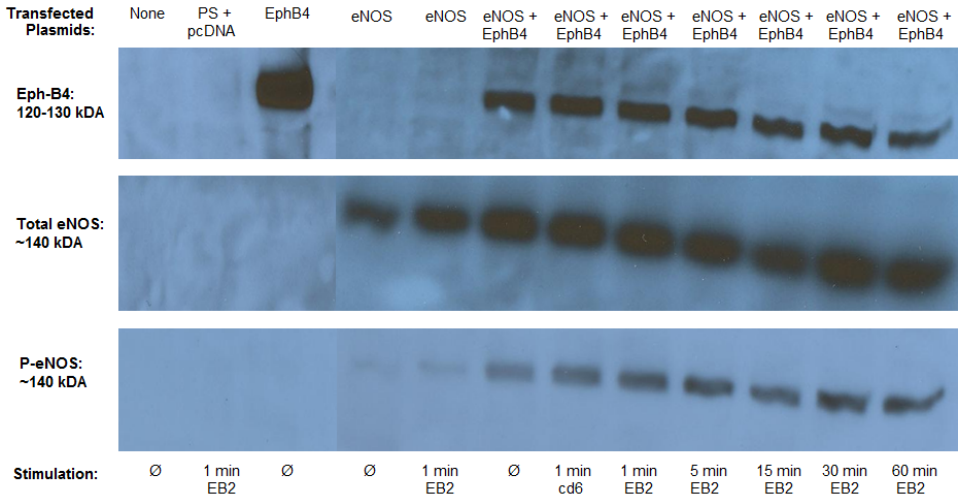

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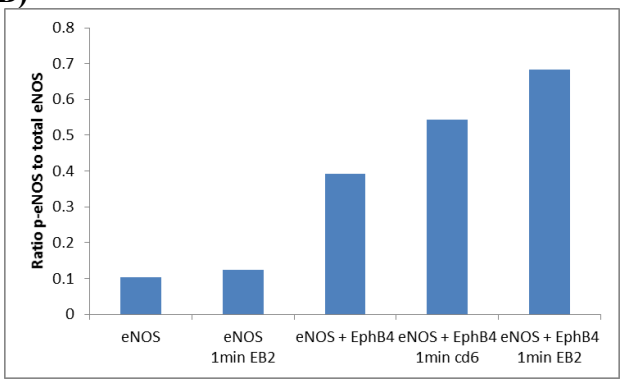

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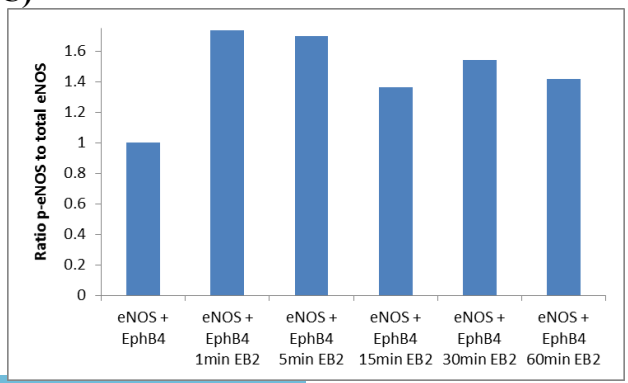

Figure 10. Eph-B4 mutant E-774 and eNOS transfection within COS cells. A) E-774, eNOS, or both were transfected into COS cells, stimulated with designated ligand, and cell lysates were taken for Western blot to measure E774, total eNOS, or phospo-eNOS. **B)** Densitometry quantification of ratio of p-eNOS to total eNOS. **C)** Densitometry quantification of ratio of p-eNOS to total eNOS, normalized to ratio in unstimulated samples. PS = P-Shuttle (vector control for E-774 plasmid). pcDNA = pcDNA13 (vector control for eNOS plasmid). EB2 = Ephrin-B2/Fc. Cd6 = cd6/Fc (control for Ephrin-B2/Fc).

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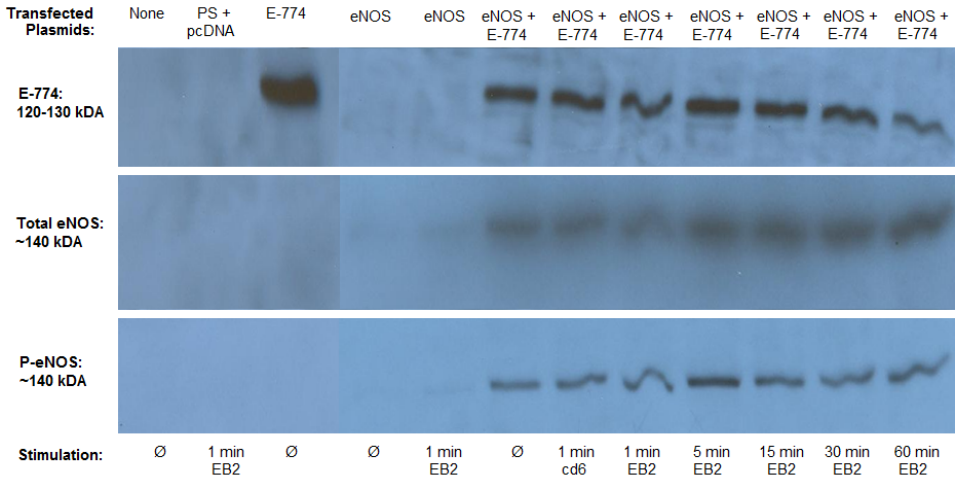

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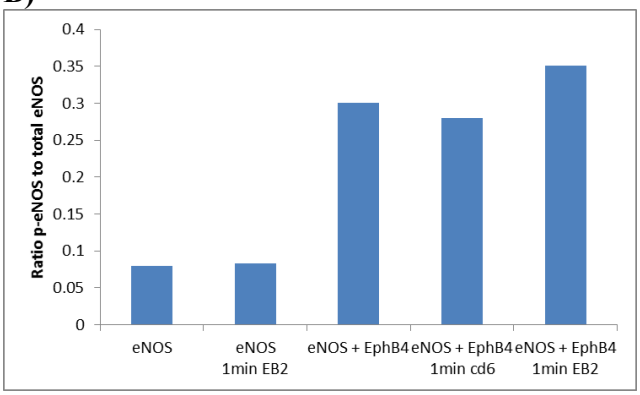

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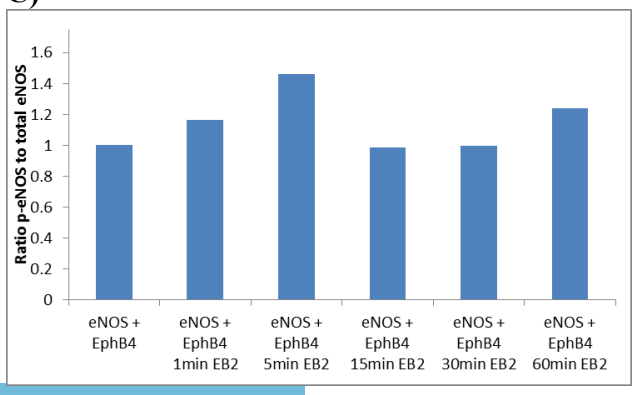

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