≷ *Review Article* ≷

Improving the Outcome of Vein Grafts: Should Vascular Surgeons Turn Veins into Arteries?

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Autogenous vein grafts remain the gold standard conduit for arterial bypass, particularly for the treatment of critical limb ischemia. Vein graft adaptation to the arterial environment, i.e., adequate dilation and wall thickening, contributes to the superior performance of vein grafts. However, abnormal venous wall remodeling with excessive neointimal hyperplasia commonly causes vein graft failure. Since the PREVENT trials failed to improve vein graft outcomes, new strategies focus on the adaptive response of the venous endothelial cells to the post-surgical arterial environment. Eph-B4, the determinant of venous endothelium during embryonic development, remains expressed and functional in adult venous tissue. After surgery, vein grafts lose their venous identity, with loss of Eph-B4 expression; however, arterial identity is not gained, consistent with loss of all vessel identity. In mouse vein grafts, stimulation of venous Eph-B4 signaling promotes retention of venous identity in endothelial cells and is associated with vein graft walls that are not thickened. Eph-B4 regulates downstream signaling pathways of relevance to vascular biology, including caveolin-1, Akt, and endothelial nitric oxide synthase (eNOS). Regulation of the Eph-B4 signaling pathway may be a novel therapeutic target to prevent vein graft failure.

Keywords: vein graft, ephrin-B2, Eph-B4, vessel identity

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Introduction

Arterial stenoses and occlusions contribute to ischemic cardiovascular diseases, the leading cause of death worldwide. Bypass surgery using vein grafts as a conduit around these lesions has developed as the mainstay approach to reperfuse the ischemic organs and tissues ever since Kunlin first described the use of autogenous veins as grafts for arterial repair in the 1940's.[1–5] Besides autologous veins, numerous alternative prosthetics such as Dacron and polytetrafluoroethylene have been developed and used as alternative conduits when vein grafts are not available. However, the mid- and long-term patency rates of prosthetic grafts are inferior to autogenous vein grafts, and therefore, autogenous saphenous vein grafts remain the gold standard for bypass surgery, particularly in the treatment of critical limb ischemia.[6–8]

During surgical creation of the vein bypass, the saphenous vein is separated from its physiological environment, necessarily inducing injury.[9,10] After harvest of a reversed vein graft, the vein loses blood flow and pressure within its lumen, and then typically is given a stretch injury as it is checked for leaks by manual application of high pressure for dilation. The harvested vein is also exposed directly to cold temperature of the air-conditioned operating room. Marking dye is often used on the outer surface to prevent twisting of the vein, which injures the vein wall and is associated with altered venous cell migration and proliferation.[11,12] In situ vein grafts require valve destruction with a valvulotome, directly producing intimal injury. Most importantly, the implanted vein is then exposed to arterial flow, with pressure, shear stress, and oxygen content distinctly different from that within the venous environment, effectively producing an injury similar to an ischemia-reperfusion mechanism. The vein graft responds to the surgical injury and the arterial environment by integrating these multiphasic stimuli, typically resulting in favorable adaptation; however, in 20–30% of cases the vein graft cannot adapt successfully, with poor clinical consequences (**Fig. 1**).
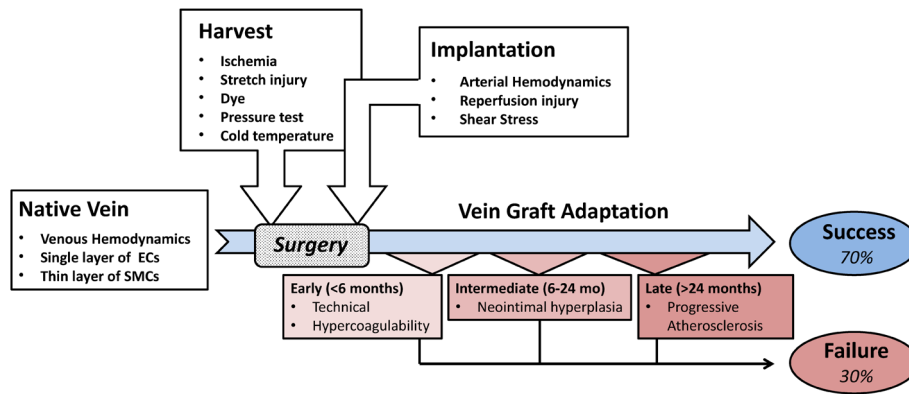
Fig. 1 Time-course of vein graft adaptation. After a harvested autologous vein graft is surgically implanted into the arterial environment, vein graft adaptation with positive remodeling and wall thickening leads to successful clinical results. In some cases, vein graft failure occurs in early, intermediate, and late periods with distinct temporal patterns of pathogenesis. EC: endothelial cell; SMC: smooth muscle cell; NIH: neointimal hyperplasia.

Peripheral artery bypass failure can be classified into early, intermediate, and late failure. Early failure occurs within 6 months after implantation, and most commonly within one month, mainly due to technical factors, hypercoagulability, and compliance mismatch.[13] Intermediate failure occurs between 6–24 months after surgery and is mainly caused by neointimal hyperplasia (NIH); it is the most common etiology of vein graft failure. Late failure generally occurs after 24 months and is typically associated with progressive atherosclerosis (**Fig. 1**). To reduce the incidence of vein graft failure and thus achieve improved long-term outcomes after vein graft implantation, we need to understand the vein graft's response to the arterial environment.

Vein Graft Adaptation

In the first report of vein graft bypass for arterial repair, Kunlin observed venous wall thickening after an initial phase of progressive dilation, calling this adaptive process "arterialization".[2] Dilation of vein grafts is described as a shear-dependent early response that occurs in the first month after implantation[14]; the endothelial cells are critically important in transducing the shear stress signal to the rest of the vessel wall.[15] The thickening of the vein graft wall is characterized by accumulation of smooth muscle cells (SMC) and extracellular matrix components, similar in mechanism to the neointimal hyperplasia that forms after injury of arterial intima.[16–18] Outward remodeling, e.g., increased diameter, and wall thickening are considered to be essential in clinically successful human vein grafts.[14,19,20]

It is likely that all arterial bypass grafts will develop NIH, given enough time,[9,10] not just vein grafts, but even prosthetic grafts that necessarily lack an antithrombotic endothelium.[21] Vein grafts are deprived of their vasa vasorum during surgical harvest, leading to relative hypoxia and possibly reduced energy source; this adventitial injury induces the release of the inflammatory cytokines from SMC.[22] Immediately after transplantation into the arterial environment, the venous wall is exposed to pulsatile flow with higher magnitudes and altered patterns of shear stress, injuring the vein graft endothelium; the responses to these environmental changes initiate vein graft adaptation, and may ultimately initiate NIH. Subsequent platelet aggregation, recruitment of the surrounding cells, and leukocyte migration with inflammatory responses contribute to the adaptive process.[23] Vessel wall thrombus after endothelial injury also induces SMC proliferation.[17] Later in the adaptive process, SMC migration and proliferation as well as extracellular matrix deposition directly contribute to remodeling but may eventually progress to NIH; the difference between wall thickening as a necessary adaptation process and the progressive pathological NIH that results in graft failure remains uncertain.[24]

Since SMC play an essential role in vein graft adaptation as well as formation of NIH, the initial approach used to overcome vein graft failure in the seminal PREVENT trials was by regulation of SMC accumulation in the vein graft wall. Since the transcription factor E2F plays a pivotal role in the coordinated transactivation of cell cycle-regulatory genes that ultimately regulate SMC proliferation, double-stranded DNA with high affinity for E2F was introduced in vivo as a decoy to bind E2F and block the activation of genes mediating cell cycle progression and intimal hyperplasia; E2F decoy successfully inhibited vascular SMC proliferation in a rat carotid injury model.[25] In a single center randomized controlled trial, intraoperative gene therapy using E2F decoy was tested in 41 patients undergoing infrainguinal arterial bypass with vein grafts;

in those patients receiving the E2F decoy, there were fewer graft occlusions, revisions or critical stenosis compared to the control group at 12 months.[26,27]

Despite these promising initial results, the subsequent multicenter randomized trials, PREVENT-III and PREVENT-IV, resulted in disappointing failure. The PREVENT-III study, in patients with critical limb ischemia, showed no significant difference between the groups in the primary end points, time to nontechnical index graft reintervention or major amputation due to graft failure.[8] The PREVENT-IV study, in patients having isolated coronary arterial bypass graft (CABG) surgery with at least two vein grafts, also failed; the primary end point, angiographic vein graft failure ($\geq 75\%$ vein graft stenosis) occurring 12 to 18 months after surgery, showed no significant difference between the groups.[28] These results suggest that mechanisms of vein graft failure are more complex than just SMC proliferation,[8,28,29] warranting further exploration.

Several preclinical studies have manipulated various intracellular signaling pathways that regulate vein graft NIH (**Table 1**). ERK is an important member of the MAPK pathway that plays an essential role during vein graft adaptation, and ERK inhibition decreased medial cell proliferation in canine vein grafts.[30,31] Statins inhibit Rho that, with pulsatile stretch, accelerates eNOS expression; pravastatin treatment reduced NIH in rabbit vein grafts.[32,33] Cyclic adenosine monophosphate response-element binding protein (CREB), a nuclear transcription factor, regulates the expression of genes essential for cell proliferation and differentiation, and is activated by a wide range of extracellular stimuli through distinct signaling pathways[34]; CREB dominant negative plasmid (KCREB) significantly repressed NIH in a mouse model.[35] Mitogen Activated Protein Kinase Activated Protein Kinase II (MAPKAP2, MK2) is an intracellular kinase that stimulates CREB transcriptional activity; MMI-0100, MK2 inhibitor peptide, prevented murine vein graft thickening.[36] Similarly, administration of Nogo-B also reduced NIH in a porcine model.[37] Although these studies were successful in animal models, no strategy has translated into clinical use for patients; issues with translation include unknown and species-specificity of the mechanisms.[38]

Molecular Fingerprints of Arteries and Veins

A novel strategy to combat vein graft failure focuses on membrane-bound signaling of venous endothelial cells in response to the arterial environment. Arteries and veins are anatomically distinguishable in the mature circulatory system. Arteries have a large diameter with a thick wall, exposed to high pressure and pulsatile flow of the highly oxygenated blood transported from the heart to peripheral tissues. Veins, on the other hand, work as a blood reservoir exposed to low pressure and relatively continuous flow towards the heart. The flexible thin wall of veins contributes to the adaptation to variable blood volume and their valves act to avoid blood reflux.

Histologically, all blood vessels have the same three-layer wall morphology: an internal intima, a media, and an external adventitia. These three layers are separated by the internal and external elastic laminae, at least in humans. Both the arterial and venous intima similarly consist of a single layer of endothelial cells lining the lumen of the vessel; however, the venous media has a significantly thinner layer of cellular and fibrous components, including circular smooth muscle cells that may contain collagen and some fibroblasts.

During embryogenesis, differentiation of undifferentiated cells into arterial or venous fate is regulated by the VEGF-delta-notch-Ephrin-Eph pathway (**Fig. 2**). Arterial differentiation of endothelial cell progenitors is initiated by activation of sonic hedgehog (Shh), a transcription factor that induces VEGF signaling; VEGF then stimulates its receptor VEGFR and co-receptor NP-1, which stimulate the Delta-Notch pathway in the endothelium,[39,40] with delta-like ligand 4 (Dll4) ligand being one the first identified arterial markers.[41] Dll-Notch then stimulates the arterial fate pathway by causing increased ephrin-B2 expression with simultaneous suppression of Eph-B4 expression; thus, Dll-Notch prevents acquisition of a venous fate.[42] Interestingly, venous differentiation is not just a "default" pathway, but is also under active control; in cells destined to become veins, chicken ovalbumin upstream promoter transcription factor 2 (COUP-TFII) suppresses Notch and ephrin-B2,[43] allowing expression of Eph-B4 and thus acquisition of venous identity.

Eph, named after its overexpression in a human

Table 1 Preclinical studies manipulating intracellular signaling to control neointimal hyperplasia in vein graft models

Mechanism	Treatment	Vein graft model (references)
ERK-1/2	ERK-1/2 inhibitor	Dog[30,31]
PTEN	PTEN adenovirus	Dog[84]
mTOR	Rapamycin	Mouse[85,86]
Rho	Statin	Rabbit[32,33]
CREB	KCREB	Mouse[35]
MAPKAP2	MMI-0100	Mouse[36]
Nogo-B	Nogo-B adenovirus	Pig[37]

PTEN: phosphatase and tensin homolog; CREB: cAMP responsive element-binding protein; KCREB: a CREB dominant protein; MAPKAP2: mitogen activated protein kinase activated protein kinase II; MMI-0100: MAPKAP2 inhibitor peptide; Nogo-B: neurite outgrowth inhibitor protein B
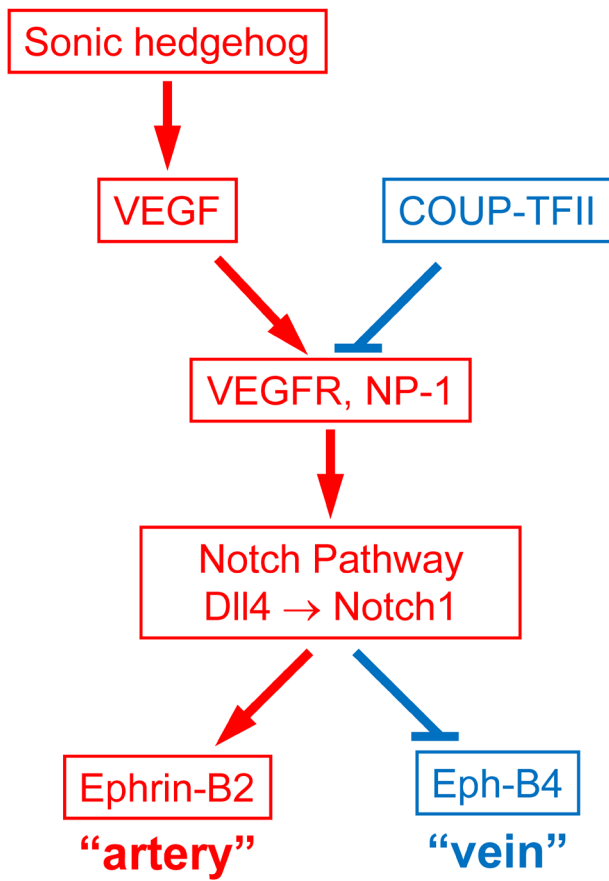
Fig. 2 Current model of arterial-venous specification in the embryo. Arterial differentiation (red): Shh regulates arterial differentiation by inducing VEGF expression in endothelial cells; notch signaling acts downstream of VEGF, inducing expression of arterial-specific marker ephrin-B2 with simultaneous inhibition of the venous-specific marker Eph-B4. Venous differentiation (blue): COUP-TFII actively blocks expression of the VEGFR; subsequent inhibition of the notch cascade prevents expression of ephrin-B2 while simultaneously stimulates Eph-B4 expression. Shh: sonic hedgehog; COUP-TFII: chicken ovalbumin upstream promoter transcription factor 2; VEGFR: VEGF receptor; NP-1: neuroplilin-1; Dll: delta-like ligand.

erythropoietin-producing hepatocellular carcinoma cell line, was first discovered in a human cDNA library screen for sequences homologous to the tyrosine kinase domain of a viral oncogene.[44] Eph receptors constitute the largest of the 14 families of tyrosine kinase receptors. Ephs, activated by their plasma-membrane-bound ligands ephrins, have many important functions during development and adulthood.[45] Unlike the majority of receptor tyrosine kinases, bidirectional signaling can originate from both the ephrin ligand and the Eph receptor. Since both the ephrin ligand and the Eph receptor are tethered to the plasma membrane, the Eph-ephrin system seems to mediate cell-to-cell interactions directly, rather than via long-range communication.[46–48] For example, the Eph-ephrin system

contributes to vascular development, axon guidance, cell migration and tissue boundary formation.[45,47] Ephrins can be divided into two subclasses, ephrin-A and ephrin-B, depending on their structural characteristics. Correspondingly, Eph receptors can be divided into Eph-A and Eph-B subclasses, based on their binding affinity to ephrins.[49]

Members of the Eph-B subclass are transmembrane proteins.[49,50] Remarkably, ephrin-B2 is specifically expressed by arteries while Eph-B4, one of its receptors, shows specific expression in veins.[48,51] Ephrin-B2 acts both as a ligand and simultaneously as a receptor for Eph-B4. These reciprocal Eph-ephrin signaling pathways are active in endothelial cells at the arterial-venous capillary interface, and are critical for angiogenic remodeling and vessel development in the embryo.[52,53] Endothelial cells expressing the arterial marker ephrin-B2 have limited ability to migrate ventrally, whereas those expressing the venous marker Eph-B4 preferentially move into the cardinal vein.[54] Eph-B4 is also a critical regulator of early lymphatic vascular development, mutations in which can cause lymphatic dysplasia.[55]

This specific expression pattern in arterial and venous endothelial cells persists into adulthood.[56] Interestingly, as development proceeds, ephrin-B2 expression progressively extends from the arterial endothelium to surrounding SMC and pericytes, suggesting that ephrin-B2 may play an important role in adult neovascularization.[57,58]

Venous Identity is Lost in Vein Grafts

Vein graft adaptation to the arterial environment involves wall thickening, fibrosis and subendothelial proliferation.[59] Venous adaptation has been called, historically, "arterialization" of the vein.[2,60] However, during rat vein graft adaptation, both Dll4 and Notch4 expression are downregulated, suggesting that "arterialization," e.g., acquisition of arterial markers, is not the correct terminology.[20,61]

To determine how vessel identity is regulated during vein graft arterialization, we analyzed the pattern of ephrin-B2 and Eph-B4 expression in veins implanted into the arterial environment.[20] Patent human saphenous vein grafts explanted from cardiac donors or limbs needing amputation showed reduced expression of Eph-B4 compared with native veins; Ephrin-B2, typically expressed at low levels in saphenous veins, was not induced in patent vein grafts. Similarly, in a rat vein graft model, which used jugular veins reversely interposed into carotid arteries, there was diminished Eph-B4, without increased ephrin-B2, compared with the native vein. A mouse vein graft model also showed similar findings, with reduced Eph-B4 expression and lack of ephrin-B2 induction.[61] Similar results were also demonstrated in ex vivo studies examining human saphenous veins exposed to increased magnitudes
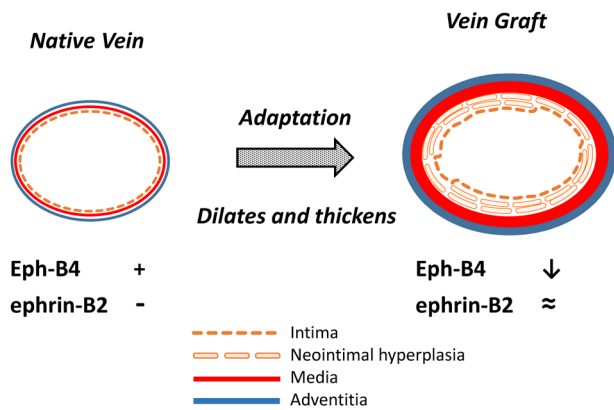
Fig. 3 The adapting vein graft dilates and thickens with decreased Eph-B4 expression and unchanged ephrin-B2 expression.

of shear stress.[62,63]

These consistent results, in 3 in vivo models and an ex vivo model, demonstrate that vein graft adaptation results in loss of venous identity, but not in a gain of arterial identity (**Fig. 3**). Moreover, these studies imply that embryonic markers of vessel identity are plastic in adults and selective regulation of those markers may be capable of modifying the course of vein graft adaptation.

Manipulation of Vessel Identity

Based on these observations, we hypothesized that Eph-B4 regulates vein graft adaptation by inhibiting venous wall thickening; we also speculated that successful inhibition of wall thickening might reduce or delay neointimal hyperplasia and thus might be a reasonable strategy for clinical translation to improve the long-term outcome of vein grafts.

To test this hypothesis, we examined the effects of altered Eph-B4 signaling in a mouse model of vein graft adaptation.[61] In this mouse model a thoracic IVC is transplanted into an infrarenal abdominal aorta of another mouse; vein graft wall thickness increased gradually up to 6 weeks after implantation with late positive remodeling by 12 weeks, recapitulating human vein graft adaptation. Mouse vein grafts also showed decreased Eph-B4 and no change in ephrin-B2, for both gene expression and protein, in the same pattern with human vein grafts. Administration of ephrin-B2/Fc, the soluble ligand of Eph-B4, induced Eph-B4 phosphorylation in venous endothelial cell (EC) in vitro, e.g., ephrin-B2/Fc activates Eph-B4. In vivo, stimulation of Eph-B4 signaling in vein grafts promoted retention of Eph-B4 in EC and, to a lesser extent in SMC, and maintained thin venous walls. Conversely, vein grafts derived from Eph-B4 heterozygous mice, e.g., with genetically diminished Eph-B4 signaling, had thicker walls, with increased layers of SMC. These results show that Eph-B4

is active in adult veins and that Eph-B4 regulates vein graft remodeling; in addition, these results suggest that vein graft failure might be prevented by stimulation or retention of venous identity, a novel therapeutic strategy. To further understand mechanisms of Eph-B4-dependent venous remodeling during vein graft adaptation, we examined some of the downstream pathways that mediate Eph-B4 functions.

Downstream Effectors of Eph-B4 Signaling

Caveolin-1

Caveolae are distinct flask-shaped invaginated structures that are located along the plasma membrane. They are recognized in the surface of many cell types including endothelial cells, and serve as signaling platforms. For example, Eph receptors enhance their signaling within caveolae. Caveolin-1 (Cav-1) is a major structural protein of caveolae in EC and is involved in mechanotransduction of dynamic shear stress changes by interacting with several signaling proteins, such as eNOS, ERK 1/2, and Eph receptors.[64–66]

To determine a mechanism of how Eph-B4 limits vein graft wall thickness, we examined Cav-1 and Eph-B4 interaction during vein graft adaptation. Eph-B4 stimulated phosphorylation of Cav-1 in EC, and phosphorylation of Cav-1 in vein grafts was correlated with Eph-B4 phosphorylation status; after activation of Eph-B4, colocalization and interaction of Eph-B4 and Cav-1 was detected. EC derived from Cav-1 knockout mice showed reduced Eph-B4-induced cell migration, and the thickened vein grafts derived from Cav-1 knockout mice were unresponsive to Eph-B4 stimulation. Vein grafts derived from mice with Cav-1 specifically reconstituted in their EC had greatly reduced thickness compared with those from Cav-1 knockout mice. These results suggest that Cav-1 mediates Eph-B4 signaling during venous adaptation, and that Eph-B4 regulation of vein graft thickening depends on endothelial Cav-1.

Akt

Phosphoinositide 3-kinases (PI3Ks) are protein and lipid kinases activated by different classes of membrane receptors; Akt is a major downstream effector of PI3K signaling.[67] Akt activates by phosphorylation, and phospho-Akt regulates a network of downstream effectors, such as eNOS; the PI3K–Akt pathway has critical roles in regulating diverse cellular functions, such as cell survival, proliferation, and metabolism. PI3K is a potential Eph-receptor binding partner; in particular, PI3K binds to the EphA2 receptor.[68] Migration and proliferation of human endothelial cells induced by activated Eph-B4 was inhibited in the presence of PI3K or Akt-inhibitors, suggesting that the PI3K–Akt pathway plays one of the central roles
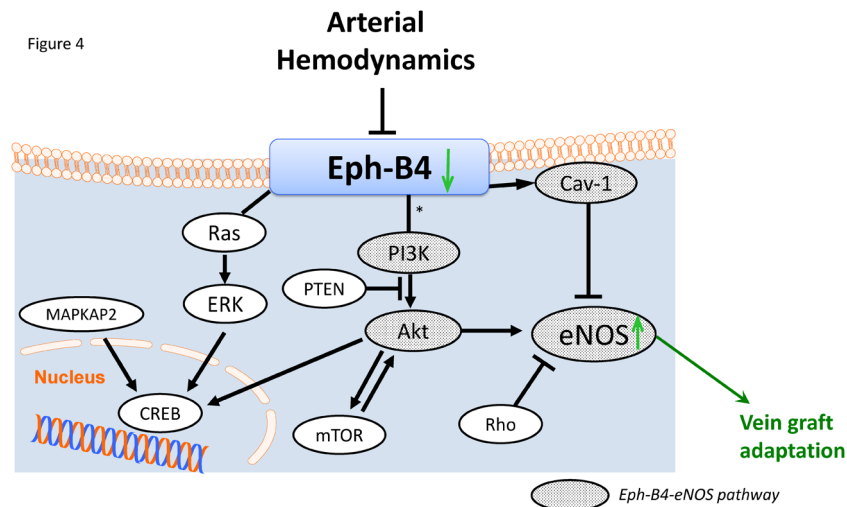
Fig. 4 Arterial hemodynamics inhibit Eph-B4 expression, leading to endothelial nitric oxide synthase (eNOS) activation. A subset of molecules interacting with Eph-B4–eNOS pathway may be therapeutic targets to regulate vein graft adaptation. Green arrows show changes with vein graft adaptation. *Discordant data between in vitro and in vivo models.

in Eph-B4 signaling in endothelial cells and vascular remodeling.[69] In vitro, decreased Eph-B4 leads to activation of Akt; however, vein grafts had increased expression of both phosphorylated and total Akt compared with native veins but no change in Akt phosphorylation.[70] These results suggest that the PI3K–Akt pathway also regulates vein graft remodeling but the differences between in vitro and in vivo results need additional study; juxtacrine and paracrine interactions between EC and SMC during vein graft adaptation complicate the interpretation of the data. In addition, Cav-1 and Akt have interactions that also require additional study.[71,72]

eNOS

Endothelial nitric oxide synthase (eNOS) is the nitric oxide synthase isoform by which nitric oxide (NO) is produced to regulate systemic blood pressure, vascular remodeling and angiogenesis.[73] Impairment of eNOS-derived NO accelerates smooth muscle cell proliferation and migration that result in NIH.[74,75] Eph-B4 regulates NO release in endothelial cells; after ephrin-B2/Fc stimulation, eNOS phosphorylation and NO production are increased in human endothelial cells,[76] while mouse endothelial cells derived from heterozygous Eph-B4 mice have less NO release compared with WT EC.[70]

Since NO is a known regulator of vein graft wall thickness,[77] we used our mouse vein graft model to determine if eNOS is a downstream mediator of Eph-B4 signaling during vein graft adaptation.[78] Activation of Eph-B4 with ephrin-B2/Fc stimulated eNOS phosphorylation and cell migration in vitro, which was abolished with eNOS inhibition. In vivo, the decreased Eph-B4 expression that occurs during vein graft adaptation correlates with increased eNOS activity; in eNOS knockout vein grafts, venous remodeling is reduced and Eph-B4 activity is enhanced. These data suggest that Eph-B4 regulates endothelial cell functions mediated by eNOS phosphorylation and that eNOS mediates venous remodeling during vein graft adaptation.

However, the discordance of eNOS regulation by Eph-B4 between the in vitro and in vivo studies remains unresolved. Diverse mediators such as Src[79] and Rho kinase[80] may play a role in regulating the Eph-B4–eNOS pathway in vivo, inducing a more complex interaction or additional responses in different cell types. In addition, Akt directly mediates eNOS activation, leading to increased NO production in EC.[81] Increased eNOS-Cav1 interaction negatively regulates eNOS phosphorylation.[79] Co-immunoprecipitation studies show that nearly all the eNOS in endothelial cells is associated with Cav-1.[82] The Cav-1 scaffolding domain serves as an endogenous negative regulator of eNOS function.[83]

Other signaling associated with Eph-B4–eNOS pathway were manipulated to regulate NIH (**Table 1, Fig. 4**). PTEN, a downstream inhibitor of PI3K, prevented Akt phosphorylation and limits NIH by decreasing SMC proliferation.[84] Rapamycin, an mTOR inhibitor, regulated NIH via PI3K–Akt signal suppression.[85,86] Interestingly, Eph-B4 regulates the Ras/ERK pathway in EC[87] (**Fig. 4**).

Manipulation of Eph-B4 in Human Saphenous Veins

Autologous saphenous vein remains the most commonly used and durable conduit for arterial bypass. To determine whether Eph-B4 is functional in human veins, as it

is in adult murine veins, we stimulated discarded human saphenous veins with ephrin-B2/Fc.[62,76)] Eph-B4 activation was associated with reduced neointimal thickening in a human saphenous vein ring assay. Stimulation of Eph-B4 in human endothelial cells induced phosphorylation of Eph-B4 and Cav-1, and release of NO. Moreover, adventitial delivery of ephrin-B2/Fc followed by 24 hours of arterial shear stress increased endothelial Eph-B4 phosphorylation. These results show that human saphenous veins have Eph-B4 and that it is functional; this data also supports the concept that regulation of Eph-B4 may be a strategy of translational potential for human patients needing vein grafts.

Conclusions

Eph-B4, the venous determinant expressed during embryonic development, remains expressed and functional in adults. Although the normal functions of Eph-B4 in adult veins are not yet clear, loss of Eph-B4 expression during vein graft adaptation suggests that Eph-B4 regulates venous wall thickness. Strategies to alter Eph-B4 activity, or its downstream effectors such as caveolin-1, Akt, and eNOS, may be translatable as a strategy to inhibit NIH that is currently the most important mechanism of vein graft failure. However, better understanding of the factors that distinguish favorable and unfavorable venous remodeling is needed before designing the next clinical trial to prevent vein graft failure.

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Disclosure Statement

All authors have no conflict of interest.

Author Contributions

Study conception: TI, TH, TN, AD
Analysis: TI, TH, KY, JMS, BY, HH, HB, GJ, TK
Investigation: TI, TH, KY, JMS, BY, HH, HB, GJ, TK
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Critical review and revision: all authors
Final approval of the article: all authors

Accountability for all aspects of the work: all authors

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