

Enhancing blood outgrowth endothelial
cells for optimal coating of blood
contacting surfaces

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Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
in partial fulfillment of the requirements
for a degree of Doctor of Philosophy in Cellular and Molecular Medicine

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University of Ottawa
2017

ABSTRACT

Implantable cardiovascular biomaterials have been widely applied in multiple cardiovascular disorders such as coronary artery disease, heart failure, and abdominal aortic aneurysms. However the failure modes of cardiovascular biomaterials are not uncommon, which is mainly due to the complications on blood-contacting surfaces such as thrombosis, calcification, and inflammation. Endothelium locates the inner surface of vessel lumen and is a critical regulator of vascular homeostasis. However, a readily available functional autologous source of endothelium has been hard to achieve. Human blood outgrowth endothelial cells (BOECs), cultured from peripheral blood mononuclear cells are proliferative and express endothelial protein profiles and as such are a very promising novel cell source for cardiovascular biomaterials coating. Endothelial nitric oxide synthase (eNOS) is an important regulator of vascular homeostasis and loss of eNOS activity is a hallmark of endothelial dysfunction. My data demonstrated that BOECs express markedly lower eNOS protein, mRNA as well as activity levels when compared to mature endothelial cells (ECs). My first project was to use transient

transfection methods along with minicircle DNA to enhance eNOS expression levels in BOECs. Two promoters were tested in BOECs, the CMV promoter (pMini-CMV-eNOS) and the EF1 α promoter (pMini-EF1 α -eNOS). Transfection with pMini-CMV-eNOS achieved 24.8 ± 5.1 times more eNOS expression when compared to null transfected cells at 24 hours, a marked improvement over that achieved with conventional PVAX plasmid (10.2 ± 4.7 fold increase) or pMini-EF1 α -eNOS (8.2 ± 1.2 fold increase both compared to null transfected control). pMini-CMV-eNOS mediated overexpression improved cell migration and network formation. When cultured on Osteopontin (OPN) coated surfaces, transient transfection with plasmid eNOS in BOECs can markedly enhance cell spreading and adhesion to ECM modified surfaces. These results suggest that eNOS expression in BOECs is suboptimal and BOECs may be functionally improved by techniques to enhance expression of this critical homeostatic regulator.

Extracellular matrix (ECM) proteins have been shown to negatively regulate eNOS expression and NO production in mature ECs. In addition, the deposition of Col IV and Col I in BOECs is higher compared to that in mature ECs. Thus, I have proposed that the lower eNOS expression/activity in BOECs compared to mature ECs is due to higher ECM deposition. When grown on fibronectin, type I collagen, type IV collagen and laminin, significantly decreased eNOS protein in HUVECs were found compared to cells on polystyrene. Interestingly, when cultured on polystyrene, BOECs express significantly more extracellular matrix (ECM) proteins especially type I collagen compared to mature ECs. Blocking collagen synthesis significantly enhanced eNOS expression in BOECs (1.77 ± 0.41 fold increase). My results suggest that the regulation of eNOS in BOECs and

mature ECs is similar and the reduced eNOS level in BOECs may be due to their increased collagen production.

ECM proteins regulate intracellular signaling transduction primarily through integrin signaling associated with focal adhesion complexes. I have proposed that ECM proteins regulation on eNOS signaling in BOECs and mature ECs is through integrin and integrin-associated proteins. Matrix mediated eNOS downregulation was blocked by β 1 integrin siRNA and focal adhesion kinase siRNA transfection in both BOECs and HUVECs. In addition, inhibitors of actin polymerization (e.g. ROCK inhibitors and cytochalasin D) block the effect of ECM on eNOS signaling. Taken together, my results suggest that ECM proteins regulate eNOS expression via a β 1 integrin/FAK/actin polymerization dependent mechanism.

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ABBREVIATIONS

acLDL	acetylated low-density lipoprotein
ANOVA	analysis of variance
BAEC	bovine aortic endothelial cells
BH₂	quinonoid 6,7-[8H]-H ₂ -biopterin
BH₃·	trihydrobiopterin radical
BH₄	(6R)-5,6,7,8-tetrahydro-L-biopterin
BOECs	Blood outgrowth endothelial cells
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CFU-Hill	colony forming unit-Hill
cGK	cGMP-dependent protein kinase
cGMP	cyclic guanosine monophosphate
Col. I	Collagen I
Cyto D	Cytochalasin D
DHE	Dihydroethidium
DMECs	dermal microvascular endothelial cells
EBM-2	Endothelial Basal Medium-2
ECM	extracellular matrix
EDRF	endothelium-derived relaxing factor
EGF	epidermal growth factor
eNOS	endothelial nitric oxide synthase
EPCs	Endothelial progenitor cells
ePTFE	expanded-polytetrafluoroethylene
ERK ½	extracellular signal-related kinase ½
FA	focal adhesion
FAD	flavin-adenine-dinucleotide
FADH	flavin adenine dinucleotide
FAK	focal adhesion kinase
FBS	fetal bovine serum
FMD	flow-mediated dilation
FMN	flavin-monomonucleotide
FN	Fibronectin
FPA	fibrinopeptide A
G-CSF	granulocyte colony-stimulating factor
HAECs	human aortic endothelial cells
HGF	hepatocyte growth factor
HIF1α	hypoxia-inducible factor 1 α
HMECs	human microvascular endothelial cells

Hsp90	heat-shock protein 90
HUVECs	human umbilical vein endothelial cells
JASPA	Jasplakinolide
ICAM-1	intercellular adhesion molecule 1
IFN-γ	interferon γ
IGF	insulin-like growth factor
IL-3	interleukin 3
IL-10	interleukin-10
ILK	integrin-linked kinase
LAMA	Laminin
L-NAME	N ^W -nitro-L-arginine methyl ester
KO	Knockout
MACs	Myeloid angiogenic cells
MCT	Monocrotaline
MNCs	mononuclear cells
NADPH	nicotinamide-adenine-dinucleotide phosphate
NK cells	natural killer cells
NO	nitric oxide
O₂⁻	Superoxide
ONOO⁻	Peroxynitrite
OPN	Osteopontin
PAECs	pulmonary aortic endothelial cells
PAH	pulmonary arterial hypertension
PDE3	phosphodiesterase 3
PECAM-1	platelet endothelial cell adhesion molecule-1
PEG	polypropylene sulfide-polyethylene glycol
PEG-SOD	polyethylene glycol-superoxide dismutase
PET	polyethylene terephthalate
PGF	placental growth factor
PHSRN	Pro-His-Ser-Arg-Asn
POC	poly(1,8-octanediol citrate)
POLY	Polystyrene
REDV	Arg-Glu-Asp-Val
RGD	arginylglycylaspartic acid
ROCK	Rho-associated protein kinases
ROS	reactive oxidase species
RT	room temperature
RVSP	right ventricle systolic pressure
Ser	Serine

SMCs	smooth muscle cells
TEBV	Tissue engineered blood vessel
TGF-β	transforming growth factor β
Thr	Threonine
Ti	Titanium
Tie-2	tyrosine kinase with immunoglobulin-like and EGF-like domains 2
TNF-α	tumor necrosis factor α
TSP	Thrombospondin
UEA-1	Ulex lectin
VCAM-1	vascular cell adhesion protein 1
VEGFR-2	vascular endothelial growth factor receptor 2
vWF	von willebrand factor
WBC	white blood cells

ACKNOWLEDGEMENTS

First I would like to thank my primary supervisor Dr. David Courtman for his guidance on my thesis project, and precious advice on my career development. During my PhD, Dr. Courtman not only provides me knowledgeable scientific ideas but the way to be a real scientist. His criticism on scientific work helped me to develop skills on scientific independency and critical thinking, which further make me a better scientist. During the last stage of my PhD, I was very anxious about finding a job. He was always available to chat and would like to share his own experience and advice with me, which was very helpful for me to make a wise decision.

Secondly I would like to thank my supervisor Dr. Duncan Stewart for his help to my research, especially his ideas to boost my project even he doesn't have a lot of time. His research angle has taught me to not only understand the scientific problem but use the learned knowledge for actual applications in patients.

I would also like to thank all the lab mates who I have spent the last four years with. Sophie, Alex, Liang Zhang, and Felix, we always have coffee breaks and chat on anything to relax. Maria, we have shared too many, if not enough, opinions from science to life. Colin, Moe, Ketul, John, Saad, Mahmoud, Yupu, Anli, Shirley, Mirabelle, Miriel, Kenny, Dhiya, I had so much fun with you guys over the last four years (soccer, volleyball, Secrete Santa and etc). I cannot imagine how my life will be without you guys in the lab. Virgilio, Rafael, I haven't had a chance to know you guys more, but I believe you will enjoy the fun life in Stewart-Courtman lab.

Lastly, I would thank my family, my wife Rui Cao, my mom and dad, I would not have been at this stage without your supports. The most important, I would thank my little new family member small angel Riley Yuan, your arrival has embraced my life. Love you all.

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CHAPTER 1: INTRODUCTION

1.1. Cardiovascular Biomaterials

Biomaterials have been widely used in various medical applications for centuries and can range from simple structural support (sutures, screws, and plates) to complex medical devices. The application of biomaterials to the cardiovascular system has led to substantial medical breakthroughs such as the introduction of artificial heart valves and cardiac pacemakers over 50 years ago. Cardiovascular biomaterials are currently a predominant category of the clinical device market with net sales of \$20.7 billion in 2014. Many different versions of cardiovascular devices such as heart valves, stents, and vascular grafts have been developed over the last century and have been used to treat hundreds of thousands of patients who suffered from coronary heart disease, cerebrovascular disease, peripheral arterial disease, aortic aneurysm, dialysis, congenital heart defect and pulmonary embolism [1]. The success of many cardiovascular biomaterials is dependent on blood compatibility and integration with the surrounding environment where they are implanted. Two important design parameters need to be considered, (1) physical and mechanical properties such as strength and deformation, fatigue, friction and wear resistance; and (2) biocompatibility with respect to material and tissue interactions as well as maintaining haematological homeostasis (reviewed in [2]).

1.1.1. Clinically Used Cardiovascular Biomaterials

Clinically available blood-contacting biomaterials (vascular grafts, stents, and heart valves) are principally composed of either synthetic (alloys or polymers such as expanded-polytetrafluoroethylene (ePTFE, Gore-Tex) or polyethylene terephthalate (PET, Dacron)) or biological (xenogenic tissue-derived) materials [3]. Dacron was developed in 1941 and is used most commonly for aortic replacement and large diameter lower

extremity bypass surgery. PTFE was first marketed under the Teflon trademark in 1945, and microporous rapidly expanded PTFE (ePTFE) materials were developed in the late 1960's by Wilbert and Robert Gore, ePTFE is much more compliant and porous compared with its nonexpanded counterpart and as such was quickly adopted for biomaterial use. Today, ePTFE is the most commonly used graft for lower-extremity and arteriovenous bypass grafting (reviewed in [4]). However, multiple early preclinical and clinical studies demonstrated ePTFE bypass grafts fail due to thrombosis or development of neointimal hyperplasia [5-8]. This is likely due to chronic activation of the coagulation cascade at the graft surface with the ultimate healing response being modified by graft flow rate and differential compliance at the anastomotic sites all of which contribute to the formation of intimal hyperplasia. These effects are common to many implantable synthetic cardiovascular biomaterials and can lead to long-term consequences of stenosis or outright occlusion, and often necessitate life long anticoagulant therapy (e.g. warfarin) associated with severe adverse bleeding effects ([7] and reviewed in [3,4]). In contrast tissue-based xenogenic materials are more resistant to coagulation yet often fail due to degradation, calcification, or hyperplasia [9,10]. In pediatric patients especially, such issues significantly increase the chances of reoperation and subsequent mortality and morbidity [11,12]. Thus, living autologous vein grafts remain the gold standard for vascular repairs as they are both compliant and contain an intact nonthrombogenic endothelial surface. However, hyperplasia within the graft, donor-site morbidity and the need for additional surgeries can limit their potential (reviewed in [13]). Thus, many researchers in the field have long believed that an ideal blood contacting surface coating consisting of autologous endothelial cells could protect cardiovascular implants from

thrombosis, intimal hyperplasia, and calcification markedly increasing their success rate well at the same time limiting the significant risks associated with long term anti-coagulant therapies.

1.1.2. Endothelialization of Cardiovascular Biomaterials

For many years the focus of biomaterial research was the development of durable bioinert materials or coatings that when applied to the cardiovascular system would not induce inflammation or coagulation. These strategies were in part successful as evidenced by the polished pyrolytic carbon coatings developed by Bokros and used in millions of synthetic heart valves since the 1970's (for review see [14]). More recently many novel polymers such as polypropylene sulfide-polyethylene glycol (PEG), poly(1,8-octanediol citrate)(POC), and hydrophilic acrylic polymers have been investigated for cardiovascular biomaterial applications. Although these materials may represent marked improvements in biocompatibility they are far from truly bioinert and even a low level of residual thromboreactivity (as in heart valves) necessitates life long anticoagulant therapy and the inherent risks therein for patients receiving these devices (reviewed in [4,15]). To overcome this residual thromboreactivity coatings such as heparin, thrombomodulin, and tissue plasminogen activator have been explored to functionalize blood contacting biomaterial surfaces (reviewed in [4]). However, to date such functionalized surfaces have failed due to lack of efficacy or durability and the stimulation of adverse immune responses in the host [16-19]. For these reasons many investigators in the field have

abandoned the search for a bioinert surface and embraced the concept of a living cellular surface that can adequately respond to changes in the in vivo environment.

The endothelial cells, which form a monolayer lining the inner surface of blood vessels, synthesize many factors such as nitric oxide, prostacyclin, tissue plasminogen activator, thrombomodulin, heparan-sulfate, and the endothelial protein C receptor (a receptor associated with the activation of protein C) to control thromboresistance and vessel wall inflammation. The endothelium regulates vessel wall homeostasis via a complex regulation of these factors in response to both physical and biological stimulations ([20-28] and reviewed in [29]). For example, under normal physiological (laminar) flow conditions, healthy endothelial cells produce increased levels of thrombomodulin an integral membrane protein that binds to thrombin acting as a co-factor in activation of protein C (anticoagulant) yet still is able to activate thrombin activatable fibrinolysis inhibitor (procoagulant, or clot stabilizing) ([30] and reviewed in [31]). Takada and colleagues have cultured human umbilical vein endothelial cells (HUVECs) under static or laminar flow conditions and demonstrated a 244% increase in thrombomodulin in dynamic culture [32]. In contrast, under pathophysiological conditions of disturbed (non-laminar) flow, Abe and colleagues have demonstrated that the expression level of tissue factor (an activator of extrinsic coagulation), in HUVECs was increased to 2 – 3 fold compared to cells under either static or laminar flow conditions [33]. Thus the ability of the endothelium to actively respond to site specific alterations in flow patterns is fundamental to the regulation of the local coagulation cascade at the vessel wall.

The endothelium also responds to cytokine and growth factor stimulations, such as interleukin-10 (IL-10), transforming growth factor β (TGF- β), tumor necrosis factor α (TNF- α), interferon γ (IFN- γ), and interleukin 3 (IL-3), to mediate local inflammatory responses (reviewed in [34] [35]). For example, treatment with the anti-inflammatory cytokine IL-10, in HUVECs produced a 3 fold increase in endothelial nitric oxide synthase and 1.5 fold increase of nitric oxide (NO) production [36] and the steady state production of NO to both decreased inflammation and thrombosis (reviewed in [37]). Under pathophysiological condition for example treatment of TNF- α , the endothelial expressions of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion protein 1 (VCAM-1), which regulate leukocyte adhesion, are markedly increased [38-40]. Thus, healthy endothelium under physiological conditions plays an inhibitory role in leukocyte adhesion and local inflammation whereas under pathophysiological conditions, endothelium may secrete factors that promote inflammation. Therefore, unlike inert material surfaces, endothelial monolayer functionally responds to external environment and regulates vascular homeostasis and as such healthy physiological environment is critical to maintain healthy endothelial function (reviewed in [41]). Currently, much research has focused on methods to engineer a native endothelial monolayer on synthetic or tissue based material surfaces with the goal of maintaining vascular homeostasis.

Early in vitro studies have applied endothelium from various sources (e.g. aorta, pulmonary microvascular, umbilical vein, and etc.) to endothelialize synthetic vascular graft surfaces or other vascularized constructs (e.g. heart valves, artificial organs, stents) and showed EC adhesion, growth, surface coverage, and phenotype were supported on biomaterial surfaces under both static and dynamic culture [42-47]. Studies have further

evaluated the function of EC-seeded grafts under blood-flow condition [47,48]. Hedeman and colleagues have perfused non-anticoagulated blood on ePTFE grafts (6 mm) seeded with human microvascular endothelial cells (HMECs), human aortic endothelial cells (HAECs) or HUVECs. They have measured the level of fibrinopeptide A (FPA), a cleavage product of thrombin-induced proteolytic cleavage of fibrinogen, in the plasma and demonstrated that plain ePTFE graft showed slightly decreased FPA level compared to grafts seeded with various ECs after 1 minute whole blood perfusion (16 ng/mL vs. 30 ng/mL mean FPA levels for plain ePTFE and grafts seeded ECs, respectively) while after 5 minutes FPA level in plain ePTFE has markedly increased and reached significantly higher than that in EC-seeded grafts (1653 ng/mL vs. 660 ng/mL mean FPA levels for plain ePTFE and grafts seeded ECs, respectively) [47]. Although increased FPA level was obtained in EC-seeded grafts compared to plain ePTFE at early time point, the progression of coagulation cascade was prevented with the presence of ECs, suggesting that endothelial cells from various sources on vascular grafts may preserve anticoagulant property after initial exposure to blood perfusion. This in vitro study has provided key information of EC functions on vascular grafts under whole blood perfusion which provided suggestive insight of EC behaviors on graft after implantation.

1.1.3. Clinical Use of Endothelialized Cardiovascular Biomaterials

Several human trials shown in table 1 have demonstrated the success of isolating autologous endothelium from excised veins and seeding onto ePTFE vascular grafts (4 – 7 mm inner diameter) after in vitro expansion for > 20 days to support long term graft patency [49-55]. Deutsch and colleagues have reported a 9-year experience on autologous

in vitro endothelialization of infrainguinal ePTFE grafts in 100 patients. In phase I of the study, 24 patients received 27 endothelialized grafts with cells harvested from external jugular vein and 16 patients received 17 untreated grafts. The Kaplan-Meier method revealed a primary 9-year patency rate for 65% for the endothelialized group, versus 16% for the control group. In Phase 2, 76 patients received 86 endothelialized

Table 1: List of clinical studies that test autologous endothelial cell-seeded ePTFE graft

References	Description	Primary patency
Magometschnigg et al., 1992 [55]	1 year, 26 patients	primary patency 55.40%
Zilla et al., 1994 [56]	Phase I, 3 years, 49 patients	primary patency 84.70%
Leseche et al., 1995 [52]	5 years, 32 patients	primary patency 67%±39%
Meinhart et al., 1997 [49]	Phase II, 7 years, 108 patients	primary patency 66%
Deutsch et al., 1999 [51]	Phase I, 9 years, 100 patients	primary patency 65%
Laube et al., 2000 [53]	27.7 months, 14 patients	primary patency 90.50%
Meinhart et al., 2001 [50]	Phase II, 7 years, 153 patients	primary patency 62.80%
Deutsch et al., 2009 [57]	9.6 years, 318 patients	primary patency 61%

ePTFE grafts with cells harvested from cephalic vein and the 5 year primary patency rate for all in vitro endothelialized infrainguinal reconstructions was 68%, indicating that autologous endothelial cell lining improves the patency of vascular grafts, which can be further used in a clinical routine [51]. Similar patency rate was also demonstrated in other 5 – 8 years follow up studies with autologous endothelial cells lining infrainguinal or femoropopliteal ePTFE grafts [49,50,52]. Leseche and coworkers have seeded autologous saphenous vein endothelial cells on autologous whole blood or plasma pre coated femoropopliteal ePTFE grafts (7 mm) followed by implantation in 21 patients. The

Kaplan-Meier analysis revealed the primary patency was $95\% \pm 10\%$ at 3 months, $89\% \pm 13\%$ at 10 and 48 months and $67\% \pm 39\%$ at 53 and 76 months [52]. Notably, the patency rates of autologous endothelial cells lining ePTFE grafts are comparable to the results (60% - 70% for 10 years) obtained by living autologous vein grafts which is the current gold standard vascular graft in 10 years follow up clinical studies [58,59].

However, many drawbacks remain to limit the potential of using autologous endothelium lining vascular grafts as a general clinical practice. First, the isolation of ECs from the existing vasculature remains challenging due to the invasive nature. Current two steps strategy involves two invasive operations: 1. Isolation of autologous ECs; 2. Re-implant EC-seeded grafts. Multiple invasive surgeries may not be tolerated well in elderly ill cardiovascular patients who are the major recipient cohort for vascular grafts [60-62]. Second, the difficulty in obtaining a sufficient number of cells is also very challenging. Most of published studies have documented the gap between ECs isolation and re-implantation is at least 35 days [49-53], suggesting that this type of graft is not suitable for emergency grafting due to the long waiting period.

One obvious solution would be the use of allogenic endothelium such as HUVECs. However, it is well documented that the allograft endothelium can initiate immune rejection through presentation of alloantigens to circulating T cells, natural killer cells and macrophages (reviewed in [63]). For example, Epperson and co-workers have cultured different concentrations of purified allogeneic CD8 T cells with HUVECs and demonstrated the proliferation of all concentrations of CD8 T cells were significantly increased while they increasingly produce IL-2 and IFN- γ after allogenic EC stimulation [64]. Bielawska-Pohl and colleagues have cultured natural killer (NK) cells with human

peripheral, mesenteric lymph node, brain, lung, and skin microvascular endothelial cells and demonstrated enhanced NK cell adherence and cytotoxicity in response to all of the allogenic ECs [65]. Most interestingly results from chromium release assays demonstrated a significant increase of killed ECs from all tested organs after incubation for 4 hours with IL-2-stimulated NK cells compared to that with resting NK cells. These results indicate that allogenic endothelial cells are the potential target of cells in immune system, which may likely lead to immune rejection during organ transplantation where graft endothelium recognized by host immune system. Thus, allogenic endothelial cells are hardly used as a proper candidate for seeding biomaterial implants due to initiation of immune cells and potential induction of graft rejection.

1.2. Endothelial Progenitor Cells (EPCs)

Endothelial progenitor cells (EPCs) are a subpopulation of hematopoietic cells originated from bone marrow and released into circulation with the potential to differentiate towards endothelial-like cells [66]. The concept of EPC was first introduced by Asahara and colleagues in 1997. They have reported that peripheral blood mononuclear cells enriched for CD34⁺ cells could differentiate into endothelial-like cells following culture on fibronectin in the presence of growth factors. After seven days in culture the fraction of cells co-expressing CD34 and vascular endothelial growth factor receptor 2 (VEGFR-2) increased. These cells also expressed other endothelial markers such as CD31 (platelet endothelial cell adhesion molecule-1, PECAM-1), tyrosine kinase with immunoglobulin-like and EGF-like domains 2 (Tie-2) and nitric oxide synthase synthase III (NOS III, eNOS), incorporated acetylated low-density lipoprotein (acLDL), bound Ulex (UEA-1) lectin in vitro [66]. Further studies have applied these cells in preclinical models and

demonstrated EPCs have the capacity to improve neovascularization and re-endothelialization of damaged surfaces (reviewed in [67-70]). Kalka and colleagues have transplanted human EPCs to athymic nude mice with hindlimb ischemia [71]. At 3 days post operation, limb perfusion was severely reduced in all tested groups. Over the subsequent 28 days, substantial blood flow recovery in mice receiving hEPCs returned perfusion of the ischemic hindlimb to levels that were similar to those recorded in the control nonischemic hindlimb, however, limb perfusion remained markedly depressed in mice receiving culture media. Histological data revealed that the capillary density was markedly increased in EPC-transplanted mice at days 7, 14, and 28 compared to that in control group. A study by Werner et al evaluated the role of EPCs on re-endothelialization. They have intravenously transfused Dil-Ac-LDL-labelled EPCs in a mouse model of carotid injury. After 14 days of injury induction, en face microscopy has revealed that homing of transfused EPCs was strictly restricted to the injury site and lining the intraluminal margin of the neointima and neointima formation is markedly decreased in EPCs transfused group compared to control group [72]. These studies suggest the important therapeutic potential of EPCs for cardiovascular disease, however, their derivation and culture methods have not reached consensus.

Human EPCs can be harvested and cultured from umbilical cord blood and peripheral blood of which the human mononuclear cells (hMNCs), which were isolated by a density centrifugation (reviewed in [73]). Currently, there are two major approaches for isolation and culture of EPCs from human MNCs. CFU-Hill method, described to transfer and culture unattached fraction of MNCs after 2 days of initial plating which was in an attempt to remove contaminating mature endothelial cells. These low density MNCs

plated on fibronectin coated tissue culture surfaces form adherent colonies after 5 to 9 days in Medium 199 with 20% FBS [74]. These colonies are referred to as colony forming unit-Hill (CFU-Hill). CFU-Hill cells have been shown to express CD31, CD105, CD144, CD146, von willebrand factor (vWF), and KDR (VEGF-R2) which are certainly consistent with an endothelial cell phenotype. However, they exhibit a low level of proliferative potential, express several monocytes/macrophage markers including CD14, CD45, and CD115. Although these cells are involved in stimulation and regulation of angiogenesis (reviewed in [75]), the true nature of these cells has been recently redefined by clonal analysis showing their hematopoietic origin [76,77]. Another method involves the direct culture of hMNCs on a fibronectin coated surface under “endothelial” culture conditions using growth factors such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), insulin-like growth factor (IGF). The medium is replaced every other days and non-adherent cells are washed away and adherent cells remain [71]. These adherent cells can give rise to two distinct populations of myeloid angiogenic cells (MACs) and blood outgrowth endothelial cells (BOECs) at different time points (reviewed in [75,78]). The following paragraphs will specifically focus on the characterizations of MACs and BOECs. Recent consensus statement on nomenclature of endothelial progenitors [79] have proposed to use the term endothelial colony forming cells (ECFCs) to substitute BOECs. However, the ECFCs mentioned by Medina et al are not the same cell population in my study. This discrepancy is mainly due to different culture techniques where I harvested BOECs by passaging a mixed population of monocyte, MACs and BOECs in EGM-2 overtime and only BOECs survived and grew whereas Medina et al have selectively picked single colonies from the mixed population

[79,80] and expanded cells from mixed colonies. Although BOECs may largely contain ECFCs, the non-colony forming endothelial cells also exist in BOECs and are involved in my study. Thus, ECFCs may not be a proper term to define BOECs. The nomenclature of EPCs has been vague. Figure 1 summarizes the nomenclature used in literatures and divides them into haematopoietic and endothelial lineages.

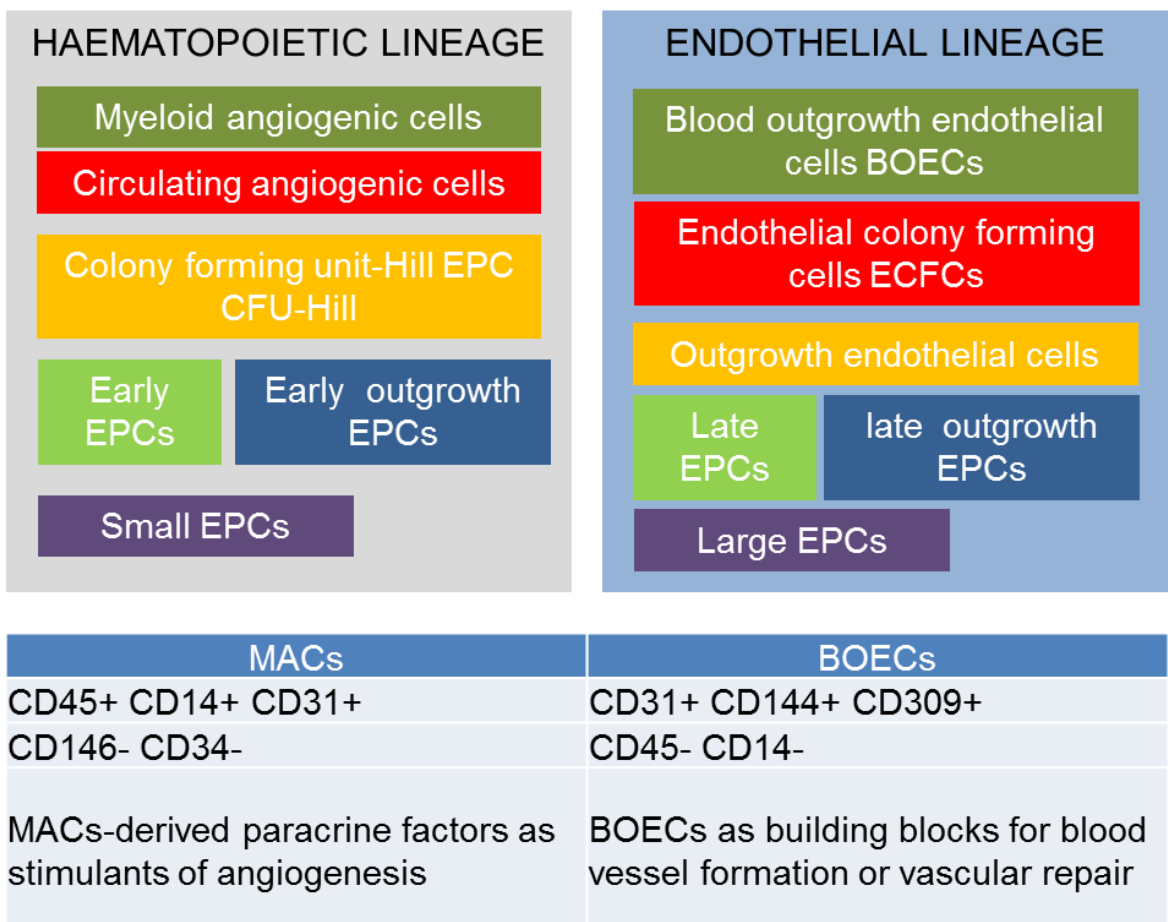


Figure 1: Complex nomenclature of cultured EPCs can be divided into two distinct categories: haematopoietic and endothelial lineages. Myeloid angiogenic cells, circulating angiogenic cells, colony forming unit-Hill EPCs, early EPCs, early outgrowth EPCs, and

small EPCs, belong to haematopoietic lineage, contain similar cell population and secrete pro-angiogenic factors for vascular repair. Blood outgrowth endothelial cells, endothelial colony forming cells, outgrowth endothelial cells, late EPCs, late outgrowth EPCs, and large EPCs, belong to endothelial lineage, contain highly similar cell population and directly involved in blood vessel formation and vascular repair. Modified with permission from [79].

1.2.1. Characterization of myeloid angiogenic cells

Myeloid angiogenic cells (MACs), emerge within one week after initial MNC plating and display heterogeneous morphology with majority of cells showing spindle shape [66,81-83]. Similar to monocytes and CFU-Hill, these cells express the pan-leukocytic marker CD45 [84,85] and the monocytic marker CD14 [86] they also display some endothelial markers vWF, CD31 and VEGFR2 [87,88]. MACs were shown to enhance neovascularization in many preclinical studies (reviewed in [73]). Kawamoto and colleagues have injected 5 rats with 10^6 MACs 3 hours after myocardial ischemia while 5 other rats received culture media. After euthanization on day 28, necropsy examination disclosed that capillary density was significantly greater in the MACs group than in the control group (290.1 ± 21.5 vs. $191.1 \pm 17.8/\text{mm}^2$ for MACs treated and control groups, respectively). Additionally, echocardiography disclosed ventricular dimensions were significantly smaller and fractional shortening was significantly greater in the MACs group than in the control group [89]. The effect of MACs was recently demonstrated to be likely through a paracrine mechanism [90-96]. Di Santo and colleagues have injected

MACs or MACs conditioned medium into rat model of chronic hindlimb ischemia. Laser Doppler analysis has revealed that both MACs and conditioned medium significantly increased hindlimb blood flow ($81.2 \pm 2.9\%$ and $83.7 \pm 3.0\%$ vs. $53.5 \pm 2.4\%$ of control). A significantly increased capillary density was obtained in both cell and conditioned medium treated groups corroborated the findings of improved hindlimb perfusion [96]. Additional studies have demonstrated that MACs secrete higher pro-angiogenic and anti-apoptotic factors including vascular endothelial growth factor (VEGF), placental growth factor (PGF), insulin growth factor (IGF-1), hepatocyte growth factor (HGF), granulocyte colony-stimulating factor (G-CSF), and IL-8 compared to mature endothelial cells such as HUVEC and human microvascular endothelial cell (HMVEC) [84,85,92,97]. Although these cells were demonstrated to play a therapeutic role in vascular repair, they are unable to form vascular network independently in matrigel tube formation assay (reviewed in [67,73]). Hur and colleagues have plated MACs alone on Matrigel and showed that these cells failed to form network-like structure after culture [84]. Additionally, gene array data has indicated that these MACs contain different gene pattern compared to mature endothelial cells such as HUVECs and HMVECs [92]. Thus, MACs are not endothelial lineage nor used as alternative candidate of mature endothelial cells for biomaterial endothelialisation.

1.2.2. Characterization of BOECs

Blood outgrowth endothelial cells (BOECs) emerge over 2 - 4 weeks in culture, form adherent colony with homogenous cobblestone morphology similar to mature endothelial

cells (ECs). These cells can achieve at least 30 population doublings in serial passaging, re-plate into at least secondary and tertiary colonies, and retain high levels of telomerase activity [84,98]. They also exhibit surface marker profiles (i.e. CD31, 133, 144, 15, 146, and KDR) and cytoplasmic vWF production similar to mature ECs [99]. Various groups have demonstrated the protein and gene expressions between BOECs and mature endothelial cells are highly similar [99-101]. For example, Medina and colleagues have used 2D-PAGE to investigate the proteomes of BOECs and human dermal microvascular endothelial cells (DMECs) and observed BOECs demonstrated over 90% of the tested protein profile in common with DMECs [99]. While MACs enhance tube formation by HUVECs, they failed to form networks when plated alone. However, BOECs can form networks when plated alone [77] and integrate into HUVEC formed networks on Matrigel [84]. When combined with synthetic or natural materials, BOECs, but not MACs, can form *de novo* functional blood vessel in vivo. Yoder et al have isolated and cultured BOECs or CFU-Hill in collagen/fibronectin gels, and transplanted them into immunodeficient mice. At 14 to 30 days, grafts were stained with either a mouse or human anti-CD31 antibody to discriminate between murine and human blood vessels. They have demonstrated that CFU-Hill cells possess myeloid progenitor cell activity, differentiate into phagocytic macrophages, and failed to produce chimeric blood vessels, confirming that CFU-Hill cells remain hematopoietic nature. However, BOECs formed chimeric vessels, which were perfused with mouse red blood cells [77], suggesting that BOECs resemble mature ECs in phenotype and can be directly involved in vessel formation.

Multiple *in vitro* studies have seeded human BOECs on surfaces of synthetic vascular grafts or vascular scaffolds and demonstrated that human BOECs adherence, growth, and phenotype are supported on various synthetic coatings (e.g. POC, polyurethane, polyglycolic-acid) under both static and flow culture [102-106]. When seeded and cultured on three-dimensional biodegradable vascular scaffolds (polyurethane foam or PGA/PH4B mesh) under dynamic culture *in vitro*, human umbilical cord blood OECs display suitable cell-to-polymer attachment and growth on both polymers while maintaining endothelial phenotype (CD31, vWF, and eNOS) after 12 days of culture [105]. Further preclinical studies have demonstrated autologous BOECs-seeding improved thromboprotective property of vascular implants [107-109]. Jantzen and co-workers seeded autologous porcine BOECs at the point-of-care in the operating room onto Titanium (Ti) tubes for 30 minutes followed by implanting into inferior vena cava of swine (n=8). After 3 days, a blinded analysis revealed that all 4 cell-seeded implants are free of clot, whereas 4 controls without BOECs were either entirely occluded or partially thrombosed [108]. Tissue engineered blood vessel (TEBV) has been used in an attempt to construct blood vessel by combining scaffold with cultured endothelial cells, smooth muscle cells, and adventitial fibroblasts. Quint and coworkers have cultured aortic smooth muscle cells on a degradable polyglycolic acid mesh scaffold in bioreactors for 10 weeks followed by decellularization. Porcine BOECs were seeded on the lumen of vessels for 3 days and these TEBVs were implanted as porcine carotid interposition grafts. After 30 days of implantation, they found that all BOEC-seeded TEBV remained patent whereas only 3/8 control vein grafts were patent. They have also determined that less neointimal hyperplasia were observed compared to control vein grafts [110]. Thus,

BOECs potentially preserve endothelial properties on vascular constructs and as such can be a proper candidate for biomaterial endothelialisation.

1.3. Surface modification to improve endothelialization

Endothelial cells adhesion to native vessel in vivo is critical to resist high shear stress from the blood flow and maintain endothelial biological functions. However, in vitro EC adhesion on uncoated synthetic vascular prostheses (e.g. ePTFE, PET) has been problematic [48,111]. Many techniques have been explored for enhancing the endothelialisation of biomaterials including surface modifications with extracellular matrix proteins, peptides, growth factors, capture antibodies, or surface functionalization of synthetic materials (reviewed in [112,113]). To date no single surface modification has demonstrated substantial clinical success.

1.3.1. Peptides

One simple way to improve endothelial cell adhesion is to modify the biomaterial surface with a simple polypeptide designed to promote cell adhesion (reviewed in [114]). For example, the cell surface integrin binding arginylglycylaspartic acid (RGD) sequence, a tripeptide composed of L-arginine, glycine, and L-aspartic acid, has been extensively studied to improve EC adhesion when linked on biomaterial surfaces ([111,115,116] [117] and reviewed in [118]). Walluscheck and colleagues have seeded human saphenous vein endothelial cells on ePTFE grafts with or without RGD-containing peptides. After 30 minutes of initial seeding, they observed a significant increase in the cell coverage on

RGD-containing peptide coated grafts compared to those without RGD (30.6% vs. 10.5%). In addition, when exposed to shear stress a significantly increased EC retention was observed on RGD-coated surfaces compared to control surfaces without RGD-containing peptides [111]. Integrins are a class of heterodimeric transmembrane proteins that regulate cell-cell and cell-matrix interactions and the RGD sequence regulates cell adhesion through several cell surface integrins: α_v (β_1 , β_3 , β_5 , β_6 , or β_8), $\alpha_5\beta_1$, $\alpha_8\beta_1$, and $\alpha_{IIb}\beta_3$ interactions (reviewed in [119,120]). Although this sequence supports cell adhesion by binding to many integrin heterodimers, this broadly non-selective binding potentially allows for the adhesion to a variety of cell types [118] including SMCs or platelets and may induce intimal hyperplasia or thrombosis after transplantation [121]. Various other peptide sequences (e.g. GFOGER, REDV) have been investigated and shown selective support of cell adhesion through single integrin binding ([122-125] reviewed in [114]). One example is Arg-Glu-Asp-Val (REDV), a peptide sequence present in the III-CS domain of human plasma fibronectin, that has been demonstrated to regulate endothelial cell adhesion through binding of integrin $\alpha_4\beta_1$ [126-128]. Massia and colleagues cultured HUVECs on REDV-treated PET grafts for 4 hours and demonstrated that endothelial cell coverage was more extensive and cell area larger than that observed on untreated PET substrates. Antibodies directed against either integrin subunit α_4 or β_1 , but not α_5 or α_v , inhibited HUVEC adhesion on REDV-PET substrates [127,128]. Thus, surface modification with peptides is a relatively simple and inexpensive way to enhance the EC adhesion and potentially improve endothelialisation. However, peptides sequence always require chemical treatment prior to surface coating, which may inevitably affect their functions such as cell adhesion (reviewed in [129]). Furthermore, the simple peptide

coating may not recapitulate complex biological events supported by extracellular matrix *in vivo* (reviewed in [130]), which all limit their use for material surfaces functionalization.

1.3.2. Extracellular matrix (ECM)

ECM is a collection of extracellular proteins and associated molecules that provide structural support and regulate cell adhesion/signaling. Many studies have used ECM molecules (fibronectin, collagens, and laminin) to coat vascular grafts and have demonstrated that ECM coatings promote endothelial cell adhesion and spreading in static culture as well as enhancing cell retention under flow condition [111,131-139]. For example, Budd and colleagues have used fibronectin in various concentrations (2, 10, 20, 50, and 100 $\mu\text{g}/\text{mL}$) to simply coat the ePTFE grafts (6 mm inner diameter) prior to cell seeding. Statistically more HUVECs were found on all fibronectin coated surfaces (seeded for periods ranging from 10 to 120 minutes under static conditions). HUVEC attachment reached 75% of cell coverage after only 10 minutes with fibronectin concentrations of 20 $\mu\text{g}/\text{mL}$ compared to only 10% cell coverage even after 120 minutes on uncoated ePTFE [138]. Further studies have evaluated the cell retention on vascular grafts under physiologically relevant shear stresses [111,115,139]. Walluscheck and co-workers have seeded adult human saphenous vein endothelial cells onto uncoated or fibronectin-coated ePTFE grafts for 30 minutes under static culture followed by medium perfusion at 3 dynes/cm^2 for 1 hour. The cell retention was determined by the percentage of cells retained on the graft after perfusion. They have demonstrated that EC adhesion after 30 minutes of seeding and cell retention after perfusion (cell coverage: 45.5% vs. 13.9% for fibronectin-coated and uncoated ePTFE, respectively) on human fibronectin-

coated ePTFE graft was significantly higher than that on uncoated graft. [111,139]. These results suggest that matrix coatings such as fibronectin may be superior to peptides, as others have shown that optimized RGD coatings yield only 30 % HUVEC coverage after 30 minutes [111]. The complex interaction of fibronectin with both the graft and cell surface may explain these differences. For example fibronectin contains additional domains that cooperate with RGD in enhancing the activation of integrin signaling. The Pro-His-Ser-Arg-Asn (PHSRN) sequence within fibronectin has been demonstrated to synergize with FN RGD sequence to enhance cell adhesion ([140-142] and reviewed in [130]). Therefore, peptides coatings hardly mimic biological events supported by ECM molecules due to its complex composition and structure (reviewed in [143,144]), and as such are hardly used as alternative for ECM substrates.

1.3.3. Surface Patterning

The topography of substrates such as ECM has been demonstrated to exert fundamental influence on cellular functions and this has been studied through surface patterning, a technique that patterns the surface with small molecules to macroscale (reviewed in [145]). Multiple studies have looked at EC adhesion on ECM substrate with different patterning themes (reviewed in [146]). Li and colleagues have cultured bovine aortic ECs on micropatterned strips of collagen matrix (60-, 30-, and 15- μm wide) in static and they have demonstrated that the spreading areas of the cells on 15- and 30- μm wide strips were 30% lower than those on 60- μm wide strips whereas more polarised focal adhesion and faster EC migration were observed in 15- and 30- μm compared to cells on 60- μm wide strips [147]. Rubenstein and co-workers have cultured human dermal microvascular

ECs for three days on patterned fibronectin (150 – 300 μm wide strips). They have demonstrated cell density increased by 4-fold on bare glass and up to 16-fold on patterned fibronectin at the end of culture [148]. Within the circulatory system, blood flow induces a surface shear stress which is a critical factor regulating cell retention and stress mediated cellular responses. Studies conducted in vitro may fail when exposed to shear due to deleterious effects on the attached cells or outright delamination of the EC layer. Zorlutuna and co-workers have seeded human microvascular endothelial cells onto linearly nanogrooved collagen films (groove widths of 332.5, 500 and 650 nm), with the grooves aligned in the direction of flow. The nanopatterns did not affect cell proliferation or initial cell alignment; however, they significantly affected cell retention under fluid flow. While cell adhesion under static culture were similar in all tested groups, the cell retention under flow were significantly higher in cells cultured on 332.5 nm and 650 nm patterned collagen films compared to that on unpatterned films ($75 \pm 4\%$ vs. $91 \pm 5\%$ vs. $35 \pm 10\%$, for 332.5 nm, 650 nm, and unpatterned films, respectively) [149]. These findings indicate that surface patterning exerts important effect on EC adhesion/retention, however, the complicated 3D ECM topographical features are hardly studied by 2D surface patterning and more sophisticated methods such as electrospinning or 3D printing are required for further investigation (reviewed in [146]).

1.4. Endothelial Nitric Oxide Synthase (eNOS)

Endothelial dysfunction is a prominent feature of cardiovascular diseases which results from reduced nitric oxide (NO) production ([150] and reviewed in [151]). This gaseous chemical is involved in a wide range of physiological and pathophysiological activities, including regulation of vessel tone, angiogenesis, platelet aggregation and adhesion, in

vascular diseases [152]. Endothelial nitric oxide synthase (eNOS) which is constitutively expressed in endothelial cells (reviewed in [152]) will be the focus of following paragraphs.

1.4.1. Physiological Functions

1.4.1.1. Vasodilation

NO was first recognized as a potent vasodilator [153-157]. Initial studies demonstrated that many stimulators such as acetylcholine, histamine, and calcium ionophore A23187 induced relaxation of rat thoracic aorta through upregulation of cyclic guanosine monophosphate (cGMP) in smooth muscle cells. This effect was completely abolished when endothelium was removed. Interestingly, sodium nitroprusside or glyceryl trinitrate induced identical relaxation and increased levels of cGMP compared to endothelial-dependent vasodilators but this regulation was independent of the presence of the endothelium, suggesting that endothelium may produce substance chemically similar to nitrovasodilators, termed endothelium-derived relaxing factor (EDRF), that directly regulates vessel dilation [153,156]. Ignarro and colleagues have then compared vascular effects of EDRF released from perfused bovine intrapulmonary artery and vein with the effect of NO delivered by superfusion over endothelium-denuded arterial and venous strips. They demonstrated that EDRF was indistinguishable from NO in that both were labile, inactivated by pyrogallol or superoxide anion, and produced comparable increased in cGMP accumulation in artery and vein. Similar reactions with hemoglobin and sulfanilic acid further confirmed that EDRF has identical biological and chemical properties as NO [154].

1.4.1.2. Inhibition of platelet aggregation and smooth muscle cells proliferation

The release of NO to vascular lumen by endothelium can also inhibit platelet aggregation and adhesion to endothelium, which subsequently prevents the release of platelet-derived growth factor that stimulates the transition of contractile smooth muscle cells (SMCs) to synthetic phenotype (reviewed in [158]). Nitric oxide activates cGMP synthesis by soluble guanylyl cyclase, an enzyme that synthesizes cGMP. cGMP further induces activation of cGMP-dependent protein kinase (cGK) and inhibition of phosphodiesterase 3 (PDE3), which promote the conversion of cyclic adenosine monophosphate (cAMP) to AMP. cAMP activates the cAMP-dependent protein kinase, a known platelet inhibitor, and leads to inhibition of platelet activation [159]. NO inhibited platelet aggregation has also been demonstrated through a cGMP-independent mechanism. Irwin and colleagues have found that ODQ, soluble guanylyl cyclase inhibitor, and KT5720 or H-89, inhibitors for PKA, failed to completely abolish the inhibitory effect of NO on platelet adhesion, suggesting the existence of cGMP-independent mechanism. They have further demonstrated that exogenous NO donors treatments dose-dependently increased S-nitrosylation of proteins in platelet, which may be of potential importance to NO-mediated regulation of platelet function [160].

1.4.1.3. Angiogenesis

Angiogenesis depicts the formation of new vessel from existing blood vessels. This process usually involves proliferation, migration, and differentiation of endothelial cells. Endothelial NO synthase was extensively investigated to improve postnatal angiogenesis (reviewed in [152,161]). Namba and colleagues have injected eNOS cDNA into rat with

ischemic hindlimb and demonstrated that both peripheral blood flow and capillary number was significantly increased 4 weeks after transfection and this effect was completely abolished by the treatment with NOS antagonist N^W-nitro-L-arginine methyl ester (L-NAME) [162]. In a 3D fibrin model, stimulated NO production in HUVECs by VEGF or FGF increased capillary-like structures formation. This effect can be completely blocked by the L-NAME [163,164]. Similar effects have been observed in vitro using substance P or transforming growth factor β (reviewed in [165]). Although these studies indicate the important role of eNOS in angiogenesis, the precise mechanism has not been elucidated. Previous study has demonstrated that eNOS may promote angiogenesis through enhancing cell migration. Murohara et al., have found that L-NAME but not its inactive enantiomer D-NAME inhibited endothelial cell sprouting from the scratched edge of the cultured bovine aortic endothelial cell monolayer. Boyden chamber assay has revealed that L-NAME inhibited endothelial cell migration but not proliferation [166]. These findings were further supported by studies performed in our lab. Ward and colleagues have transfected human eNOS into MACs and demonstrated that eNOS transduction significantly increased cell migration and network formation but not proliferation [167]. Thus, enhanced eNOS expression may improve in vitro angiogenesis through cell migration but not proliferation. As a consequence of these important findings described above, research into NO led 1998 Nobel Prize for its discovery as a cardiovascular signalling molecule.

1.4.2. Regulation of eNOS activity

1.4.2.1. eNOS Activation

Under normal physiological conditions, eNOS (also referred to as type III NOS) catalyzes the oxidation of L-arginine to L-citrulline and synthesizes NO, using (6R)-5,6,7,8-tetrahydro-L-biopterin (BH₄) and flavin adenine dinucleotide (FADH) as essential co-factors and molecular oxygen and reduced nicotinamide-adenine-dinucleotide phosphate (NADPH) as co-substrates. During nitric oxide synthesis, eNOS monomers are able to transfer electrons from reduced NADPH, to flavin-adenine-dinucleotide (FAD) and flavin-mononucleotide (FMN). The elevated level of calcium induces binding of calmodulin to eNOS monomers, which enhances electron transfer within the reductase domain. In the presence of haem, eNOS can form a functional dimer. Haem is essential for the inter-domain electron transfer from the flavins to the haem of the opposite monomer. When enough substrate L-arginine and cofactor BH₄ are present, the haem on eNOS monomers are coupled and O₂ is reduced to NO synthesis (reviewed in [158]) (Figure 2). Besides induction of calcium flux, eNOS can be activated by the recruitment of heat-shock protein 90 (Hsp90) [168-170]. Previous study has demonstrated that eNOS and Hsp90 form complex in both bovine lung microvascular endothelial cells and rat aorta. NOS activity assay has revealed that overexpression of Hsp90 in endothelial cells resulted in increase of eNOS activity. The induction of VEGF, histamine or shear stress can promote a rapid recruitment of Hsp90 to eNOS complex and stimulate eNOS activity. In addition, this agonist-induced NO production was inhibited when Hsp90 signalling was blocked, indicating the important role of Hsp90 in eNOS activation [168].

The eNOS phosphorylation or dephosphorylation on several serine (Ser), threonine (Thr), and tyrosine residues has also been proposed to be involved in eNOS activation (reviewed in [171]). Various eNOS agonists such as shear stress, VEGF, estrogen, bradykinin phosphorylate eNOS at multiple sites and induce enzyme activation (reviewed in [158]). Dimmeler and colleagues have demonstrated that HUVECs exposure to shear stress result in more than 2 fold increases in eNOS phosphorylation of S1177 residue. Mutation of S1177 in COS cells transfected with wild-type eNOS prevented shear-stress-induced nitrite accumulation [172]. Michell and co-workers have treated HUVECs with VEGF and demonstrated a transient increase in S1177 phosphorylation and a decrease in T495 phosphorylation. Human eNOS S1177 and T495 correspond to bovine eNOS S1179 and T497, respectively. Treatment of bovine aortic endothelial cells (BAEC) with phosphodiesterase inhibitor or forskolin caused dephosphorylation of T497 and enhanced phosphorylation of S1179 resulting in increased eNOS activity [173].

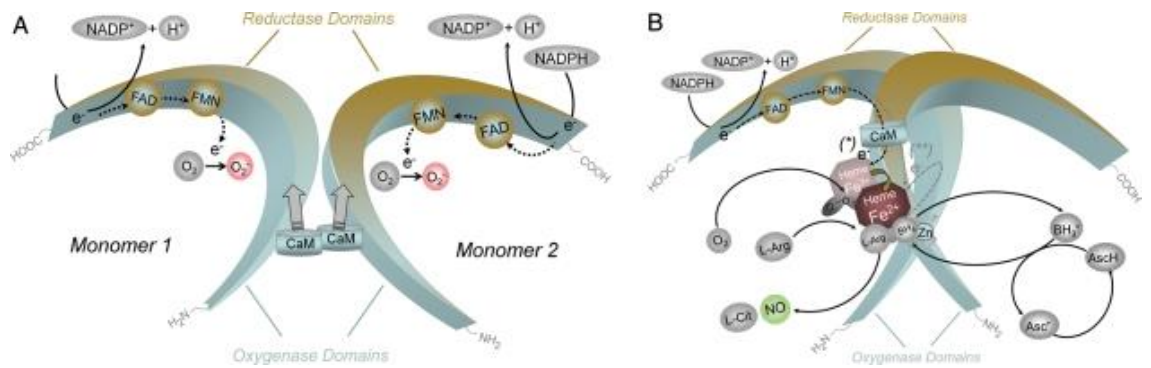


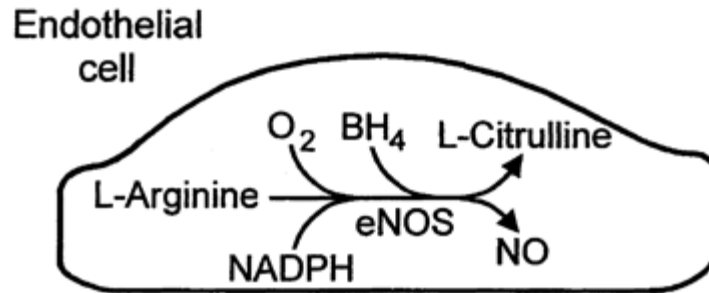
Figure 2: Schematic description of structure and catalytic mechanisms of eNOS (A) the electron transfers from NADPH to FAD and FMN in eNOS monomer and have a limited capacity to reduce molecular oxygen to superoxide. Monomers and isolated reductase domains can bind calmodulin, which enhances electron transfer within the reductase domain. (B) In the presence of haem, eNOS can form a functional dimer and allows for interdomain electron transfer from the flavins to the haem of the opposite monomer. eNOS monomers are unable to bind the cofactor BH₄ or the substrate L-arginine and cannot catalyze NO production [158]. With permission from [158].

1.4.2.2. eNOS Uncoupling

Under pathophysiological conditions, such as hypertension, hypercholesterolaemia, diabetes mellitus, cigarette smoking, etc., NADPH oxidases are upregulated and enhance reactive oxidase species (ROS) production. The increased oxidase stress converts eNOS from an NO-producing enzyme to an enzyme that generates superoxide ($O_2^{\cdot-}$). This process has been referred to as eNOS uncoupling (Figure 3). There are several mechanisms involved in this process including oxidation of NOS cofactor BH₄, depletion of L-arginine, and accumulation of endogenous methylarginines. Among these, BH₄ is of primary importance for eNOS uncoupling.

Tetrahydrobiopterin is a critical cofactor of eNOS and can be oxidized to trihydrobiopterin radical (BH_3^\cdot), which further disproportionate to the quinonoid 6,7-[8H]-H₂-biopterin (BH_2), in the presence of $\text{O}_2^{\cdot-}$ and peroxynitrite (ONOO^-) (the product of $\text{O}_2^{\cdot-}$ and NO). As a consequence, oxygen reduction by eNOS is uncoupled from NO formation, a functional eNOS is converted into a dysfunction $\text{O}_2^{\cdot-}$ -generating enzyme. Oxidation of BH_4 to BH_2 also reduces the affinity of the substrate L-arginine to NO, leading to the production of $\text{O}_2^{\cdot-}$ (reviewed in [158]). Treatment with oral BH_4 has been developed as a potential therapeutic target to reduce ROS production, eNOS uncoupling and increase NO production and improve vascular function. In the chronic hypoxia-induced pulmonary hypertension rat model, chronic BH_4 oral administration enhanced lung eNOS activity and reduced superoxide production, with a net increase in cGMP levels. This treatment has further attenuated the pressor response to hypoxia with reduced right ventricular systolic pressure and right ventricular hypertrophy [174]. Thus, eNOS can play an important role in vascular homeostasis, however, pathophysiological conditions such as turbulence flow, hyperglycemia, and hypercholesterolemia, may result in enzyme uncoupling along with superoxide production and as such a proper controlled environment is critical for eNOS activation.

"Coupled eNOS"



"Uncoupled eNOS"

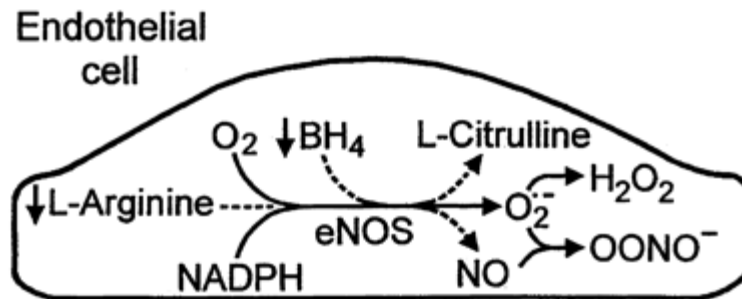


Figure 3: Schematic representation of 'uncoupling' of NO synthesis from consumption of NADPH oxidases. Under physiological conditions in healthy endothelial cells, coupled eNOS catalyzes the conversion of L-Arginine to L-Citrulline with the presence of BH₄ and produces NO. Under pathophysiological conditions, the upregulation of BH₄ oxidization by NADPH oxidases reduces eNOS coupling, which further promotes production of superoxide instead of NO. With permission from [175].

1.4.2.3. Extracellular Matrix regulation of eNOS expression and activity

Within the circulatory system, shear stress is critical not only to induce morphological changes of endothelium on the vessel wall but triggers biochemical and biological events including eNOS activation (reviewed in [176]). One of the hypotheses proposed that the eNOS regulation by shear stress is through interaction with basal extracellular matrix substrates ([177,178] and reviewed in [179]). The following paragraphs will be focused on eNOS regulation by ECM substrates.

1.4.2.3.1. ECM Regulation

Studies have previously demonstrated the tight correlation of ECM substrates and eNOS signaling in ECs [180-182]. Gonzalez-Santiago et al., cultured HUVECs on type I or type IV collagen coated surfaces. Compared to normal basal membrane ECM component Collagen IV (Col. IV), abnormal ECM protein collagen I (Col. I) in vessel walls was demonstrated to decrease eNOS expression and NO production through an integrin-linked kinase (ILK) dependent pathway. Luciferase assay further revealed that the eNOS promoter activity was decreased in HUVECs grown on Col. I compared to that on Col. IV [180]. FN and laminin (LAMA), the main basement membrane ECM components, have both been shown to negatively regulate eNOS expression and activity [181,182]. Viji and colleagues demonstrated both eNOS expression and activity levels in HUVECs were downregulated when cells cultured on FN. The reduced eNOS activity was reversed after adding p38 MAPK inhibitor (SB202190). They have further demonstrated that Ser phosphorylation was reduced when cultured on FN and this effect was abolished when p38 MAPK was blocked suggesting that FN substrate decreases eNOS activity through

reducing Ser phosphorylation via a p38 MAPK dependent mechanism [182]. This consistent negative regulation across different ECM substrates on eNOS signaling suggests the existence of an ECM substrate independent mechanism.

1.4.2.3.2. Actin Cytoskeleton

Cell binding to ECM substrates initiates integrin clustering, activates adhesion associated proteins and polymerizes actin cytoskeleton. An association between actin cytoskeleton organization and eNOS expression/activity has previously been described [183] [184] [185] [186]. Endothelial cells exhibited decreased actin stress fiber formation after dominant-negative RhoA mutant overexpression resulted in increased eNOS expression and activity. Mice treated with a Rho inhibitor or the actin cytoskeleton disrupter cytochalasin D showed increased vascular eNOS expression and activity, leading to a decrease in cerebral infarction size after middle cerebral artery occlusion [184]. Su et al., have demonstrated that incubation of purified eNOS with either monomeric or globular (G) –actin or filament (F) –actin increase the eNOS activity and the G-actin-induced upregulation was much higher than that caused by F-actin. Treatment with swinholide A, an actin filament disruptor, resulted in an increase in eNOS activity, eNOS protein content in pulmonary artery endothelial cells (PAECs). In contrast, incubation of PAECs with phalloidin, an actin filament stabilizer decreased in eNOS activity and expression [186]. These data suggests a negative regulation of eNOS signaling by actin cytoskeleton formation. However, a study by Searles et al., demonstrated the opposite effect when studying confluent ECs. They have examined the relationship between the state of actin polymerization and eNOS expression in confluent ECs and found that cytoplasmic G-actin co-localized with eNOS transcripts in confluent ECs. They have further identified

that G-actin binds the eNOS mRNA 3' untranslated region and this binding is inversely related to eNOS mRNA expression. Treatment with jasplakinolide, a potent inducer of actin polymerization and a stabilizer of actin filaments, reduced the G/F-actin ratio and increased eNOS expression [185]. This discrepancy suggests that the cell confluency may play an important role in the regulation of actin cytoskeleton on eNOS expression/activation.

1.4.2.3.3. Matricellular Proteins

Matricellular proteins are a group of non-structural proteins present in ECM and involved in intracellular regulation. Thrombospondin (TSP) is a family of matricellular proteins consist of thrombospondins 1-5 that mediates cell-to-cell and cell-to-matrix interactions. Studies demonstrated the key regulation of TSP1 on eNOS signaling [187,188]. Bauer and colleagues demonstrated that TSP1 inhibits acetylcholine-stimulated eNOS activation and agonist-driven calcium transients in ECs. In vivo data demonstrated that TSP1-null vessels have greater endothelial-dependent vasorelaxation compared with the wild type. TSP1-null arteries demonstrated less vasoconstriction to phenylephrine compared with the wild type, which was corrected upon inhibition of eNOS, indicating TSP1 negatively regulates endogenous eNOS activity and vasorelaxation [188]. Their further study has demonstrated that TSP1 activates its receptor CD47 and disrupts the interaction of CD47 and caveolin-1 and further inhibits eNOS activity [187]. Another study has proposed the relationship between TSP2 and eNOS. MacLauchlan and co-workers have found NO represses TSP2 promoter activity in vitro. Their in vivo study has shown that double-eNOS/TSP2 knockout (KO) mice were found to rescue the phenotype of eNOS KO mice. Studies in mice with knock-in constitutively active or inactive eNOS on the Akt-1 KO

background showed that eNOS activity negatively correlates with TSP2 levels. These results suggest that eNOS regulates angiogenesis by downregulating TSP2 expression [189].

1.4.2.3.4. Shear Stress

Shear stress is the force per unit area created when a tangential force (blood flow) acts on a surface (endothelium). Endothelium lining the cardiovascular system is highly sensitive to hemodynamic shear stresses that act at the vessel luminal surface the direction of blood flow (reviewed in [190,191]). Passerini and colleagues have used cDNA arrays to analyze regional EC gene expression under disturbed flow, which are believed to be areas susceptible to atherogenesis, or undisturbed laminar flow in adult porcine aorta. They identified approximately 2000 putatively differentially expressed genes between cells isolated from two regions. Within these differentially expressed genes, there was an upregulation of several inflammatory cytokines, and receptors in ECs under disturbed flow and downregulation of angioprotective genes including eNOS [192]. Many further studies have demonstrated that hemodynamic shear stress regulation of eNOS is through cell-matrix interactions which involve molecules such as integrin, c-Src/extracellular signal-related kinase $\frac{1}{2}$ (ERK $\frac{1}{2}$), focal adhesion kinase, and actin cytoskeleton ([178] [193] [194] and reviewed in [195] [190]). Loufrani and co-workers have demonstrated that flow-mediated dilation (FMD) in mesenteric resistance arteries was inhibited by either anti- α_1 integrin blocking antibodies or genetic deficiency in α_1 -integrin in mice. This effect was abolished when cells were treated with LY-294002, a PI3-kinase-Akt inhibitor, suggesting that shear stress induced FMD is regulated by α_1 -integrin/PI3-kinase/Akt/eNOS dependent mechanism [194]. When cultured under laminar shear stress,

Davis et al., have demonstrated that eNOS transcription as well as mRNA stability in bovine aortic ECs was markedly increased compared to non-shear culture condition. This effect was completely abolished by treatment with Src family inhibitors PP1 and PP2. Further study has demonstrated that this effect can also be attenuated by addition of either PD98059 or UO126, which are selective inhibitors of ERK, suggesting that shear-induced eNOS upregulation is through a c-Src/ERK dependent mechanism [178]. These results indicate the tight correlation between eNOS expression/activity and hemodynamic shear stresses and this regulation is highly dependent on cell interaction to basal substrates.

1.4.3. eNOS and EPCs

Endothelial dysfunction is a prominent feature of cardiovascular diseases which results from reduced nitric oxide production. Endothelial NOS mediates a variety of angioprotective functions including endothelial mobilization in wound healing, neovascularization, blood vessel tone, preventing thrombosis and platelet adhesion [166,196] and as such is an ideal candidate gene for restoring endothelial functional activity. MACs have capacity of vascular repair such as neointimal hyperplasia, myocardial infarction, and pulmonary arterial hypertension. MACs have been reported to contain limited expression of eNOS [86] yet these cells when genetically engineered to enhance eNOS expression can improve their therapeutic effect in treating multiple cardiovascular disorders (such as neointimal hyperplasia [197], myocardial infarction [198] and pulmonary arterial hypertension [82,199]). A study demonstrated that rat bone marrow derived EPCs transfected with eNOS are able to repair damaged lung vascular endothelium in animals induced by monocrotaline (MCT) [199]. Zhao et al.,

demonstrated in the prevention model that EPCs were able to prevent MCT injury to the pulmonary vasculature. In the reversal model, they have compared the effects of EPCs, eNOS-transfected EPCs, and saline. While EPCs were able to prevent progression of pulmonary hypertension, eNOS-transfected EPCs were able to significantly lower right ventricle systolic pressure (RVSP) and the weight ratio of right ventricle to left ventricle plus septum (RV/LV+S) levels, reduce arteriolar muscularization, and improve perfusion [199]. Other studies have looked at this enzyme in human EPCs which contain two distinct populations, MACs and BOECs [197,200]. Cui and colleagues have injected MACs or eNOS-EPCs by tail vein after balloon injury. After two weeks, computer-based morphometric analysis revealed that MACs transfused group decreased neointimal thickness to 1/2 of that in nontransfused group. Notably, eNOS-MACs can further reduce the neointimal thickness to 1/3 of that in nontransfused group. Furthermore, eNOS-MACs transplantation increased significantly endothelium-dependent vasodilation compared with MACs transplantation [197]. Kong and colleagues have transfected BOECs with eNOS and transplanted them into rabbits which were subjected to balloon angioplasty of common carotid artery. After two weeks, morphometric analysis showed a decrease of 48.3% in neointima/media ratio in control BOECs transplanted vessels and 72.1% in eNOS-transfected BOECs transplanted vessels compared with the saline control [200]. These studies demonstrated eNOS transfection improved therapeutic functions in both animal and human EPCs. Human MACs display monocytic markers and few endothelial cell markers whereas BOECs express over 99% homology of mRNA to mature ECs and markedly higher level of eNOS had been observed in BOECs compared to MACs. One study has compared the effect of human MACs and BOECs in pulmonary

arterial hypertension model. Ormiston et al., have demonstrated that in athymic nude rat, MACs prevented MCT-induced increases in right ventricular systolic pressure and right ventricular hypertrophy whereas BOECs were ineffective [82]. Although the different effect may be due to the distinct therapeutic mechanisms in two cell populations, the endogenous eNOS in BOECs may not sufficiently exert therapeutic function in PAH model. Therefore, in subsequent Chapters, I have compared the expression/activity of eNOS in BOECs and mature ECs and demonstrated possible signaling mechanisms that may explain this differential regulation.

1.5. Rationale and Hypotheses

Synthetic or metal-based cardiovascular material implants are often associated with numerous short- and long-term complications. One method proposed to create an ideal cardiovascular surface would be to engineer a native endothelial monolayer on the material surface to enhance patency and maintain vascular homeostasis. BOECs, markedly amplified and purified in culture, represent a highly proliferative autologous cell population, displaying over 99% of mRNA homology to mature ECs and as such are a promising candidate for cardiovascular biomaterial endothelialisation. Endothelial NOS is a key regulator of vascular homeostasis and mediates a variety of angioprotective functions. This enzyme has been extensively demonstrated to improve therapeutic effect of MACs in treating multiple cardiovascular diseases however, it has undergone limited testing in BOECs for cardiovascular biomaterial applications. Thus, chapters 2 through 4 of this thesis are presented as independent but highly related studies, all of which represent novel contributions for understanding the biological regulation of eNOS in

BOECs and further improving BOECs phenotype for cardiovascular biomaterials coating.

An outline of the rationale and hypothesis for each study is listed below.

Table 2: Rationale and hypotheses of each Chapter

Chapter 2	<p>Rationale:</p> <ul style="list-style-type: none"> • BOECs express markedly lower eNOS protein, mRNA as well as activity levels when compared to classic endothelial cells such as HUVECs or HAECs. • eNOS is an important regulator of vascular homeostasis in vivo and loss of eNOS activity is a hallmark of endothelial dysfunction
	<p>Hypothesis: Transient transfection methods enhance eNOS expression levels in BOECs and subsequently improve their angiogenic capacity.</p>
Chapter 3	<p>Rationale:</p> <ul style="list-style-type: none"> • ECM is a collection of extracellular molecules that provides structural support and negatively regulates eNOS signaling in HUVECs. • BOECs deposit ECM proteins that assemble to an organized weblike structure and mature ECs only express these ECM proteins intracellularly in vitro.
	<p>Hypothesis: Reduced eNOS expression is due to increased ECM production in BOECs compared to mature ECs.</p>
Chapter 4	<p>Rationale:</p> <ul style="list-style-type: none"> • ECM regulates intracellular signaling through integrin signaling associated with focal adhesion complexes.
	<p>Hypothesis: ECM molecules regulate eNOS signaling through integrin signaling associated with focal adhesion formation and actin polymerization.</p>

**CHAPTER 2: ENDOTHELIALIZATION OF
BLOOD CONTACTING SURFACES WITH
ENOS-TRANSFECTED BOECS**

Part of the results (Figs. 5, 8, and 9) in this Chapter have been published in *Biomaterials* (Yuan, Yifan, Altalhi, Wafa A, Ng, Jeannette J, Courtman, David W. Derivation of human peripheral blood derived endothelial progenitor cells for biomaterial application: the role of osteopontin surface modification and eNOS transfection. *Biomaterials*, 2013 Jun 27. pii: S0142-9612(13)00677-7. doi: 10.1016/j.biomaterials.2013.06.003.) [201] where I served as the first author. The work on OPN coating in combination with BOECs (Fig. 8) was performed by Wafa Altalhi who is the previous graduate student in the lab and the co-first author in this paper. All results (Figs. 5, 8, and 9) have been reproduced with permission.

2.1.Introduction

Derivation of EPCs from high-volume leukapheresis is a relatively safe and effective procedure even in high-risk patients and thus may represent a very useful means of obtaining sufficient progenitor cells for biomaterial applications [202,203]. Indeed, de novo transplantation of *ex vivo* conditioned autologous EPCs is being developed as a novel therapeutic strategy for the treatment of multiple cardiovascular diseases (such as myocardial infarction [198] and pulmonary arterial hypertension (PAH) [82]. If maintained in culture EPCs undergo a transition to cells of mature endothelial phenotype: growing in a cobblestone morphology, displaying contact inhibited growth, and expressing CD31, and VEGFR-2 [84]. These BOECs are a very promising novel cell source for cardiovascular biomaterials and to date has undergone limited testing for these applications [204].

Regenerated endothelium on denuded or implanted biomaterial surfaces often lack appropriate integrity and function, displaying poorly formed cell junctions and reduced expression of antithrombotic proteins [205]. eNOS mediates a variety of angioprotective functions *in vivo* including endothelial migration, neovascularization, blood vessel tone, anti-thrombosis and preventing platelet adhesion [166,206] and as such is an ideal candidate gene for restoring endothelial functional activity. Previous studies have demonstrated eNOS transfection can improve therapeutic effect of EPCs in multiple cardiovascular disease preclinical models [167,199]. However, current applications of plasmid based gene therapy are limited by inefficient transgene expression and the adverse responses to bacterial motifs. These sequences may cause undesirable effects such as the production of antibodies against bacterial proteins expressed from cryptic

upstream eukaryotic expression signals, changes in eukaryotic gene expression caused by the antibiotic resistance marker, and immune responses to CpG sequences (reviewed in [207]). Minicircle vectors are double stranded DNA of reduced size and are devoid of bacterial sequences. It has been widely used for many transfection studies (including HIF-1 α and VEGF) to enhance and prolong the transgene expression [208,209].

Many techniques have been explored for enhancing the endothelialization of biomaterials including surface modifications with extracellular matrix proteins, peptides, growth factors, capture antibodies, or surface functionalization of synthetic materials [112,113]. To date no single surface modification has demonstrated substantial clinical success. Recently, Leen et al. demonstrated that osteopontin (OPN) is an essential element for endothelial repair *in vivo*, and as such OPN surface modification may be a promising candidate for biomaterial applications [210]. Osteopontin is an RGD-containing matricellular protein [211]; it exists both as an immobilized ECM molecule and as a cytokine playing a role in both cell adhesion/migration through cell surface integrin α_v (β_1 , β_2 , or β_5) and (α_4 , α_5 , α_8 , or α_9) β_1 interactions [212] and cell signaling through interaction with CD44 [212]. *In vivo* OPN expression in injured arteries promotes increased endothelial repair and mononuclear cell incorporation likely by mediating cell adhesion to wounded areas [210,213]. *In vitro* OPN has been shown to stimulate vascular cell adhesion, migration, and survival [214]. Since OPN appears to play a role in the homing and incorporation of EPCs to the site of endothelial injury it may be a promising candidate to enhance EPC specific adhesion and function on biomaterial surfaces. Therefore, I hypothesized that transient transfection methods using minicircle DNA enhance eNOS expression levels in BOECs and subsequently improve their angiogenic

capacity. In the current study I derived and characterized BOECs from human apheresis samples, and demonstrated an increase in functional activity when BOECs are transfected with minicircle eNOS and grown on OPN coated surfaces.

2.2. Specific Aims

- To optimize a protocol to improve BOECs yield from human leukapheresis products.
- To develop optimized protocols for transient transfection of eNOS in BOECs.
- To quantitatively measure the impact of eNOS on migration, proliferation, and in vitro angiogenesis in BOECs.
- To demonstrate the synergistic effect of matricellular substrate and eNOS transfection on BOECs adhesion and spreading.

2.3. Materials and methods

2.3.1. Endothelial progenitor cell culture and characterization

Human peripheral blood monocytes were obtained by leukapheresis from 10 non-mobilized healthy donors as previously described [82]. Leukapheresis samples were collected using a COBE Spectra Apheresis System (Cobe Spectra, Gambro BCT, Denver, CO) in GMP facility at Ottawa Hospital Research Institute, 3 blood volumes were processed to obtain a final sample volume of 150 mL to 200 mL. Cre Comanita has used Biosafe Sepax system (Sepax Technologies, Newark, DE) to ficol leukapheresis samples.

I have cultured the ficol (GE Healthcare, Mississauga, Ontario, Canada) separated fraction on fibronectin (Roche Applied Sciences, Indianapolis, IN) coated flasks in supplemented Endothelial Basal Medium-2 (EBM-2) (Clonetics, Lonza, Walkersville, MD) containing 20% human serum. MACs emerged after 1 week of culture and BOECs, which represented the BOECs used throughout this thesis, emerged after 2 weeks and were used between passage 3 to 6. Cells were imaged by phase-contrast light microscopy (Nikon Eclipse, T5100) and characterized by flow cytometry (Beckman Coulter, Mississauga, Ontario, Canada), with the following primary conjugated antibodies: CD31–FITC, CD14–PE, CD45-PE, VE-Cadherin-FITC, CD34–APC (BD Biosciences, San Diego, CA), and KDR–PE (R&D Systems, Minneapolis, MN). Cells were incubated with PECAM and vWF antibodies followed by secondary antibodies conjugated with Alexa fluor 488. For detection of AcLDL uptake and UEA lectin, cells were cultured until 80% confluence followed by incubating with dil-AcLDL for 2 hours. Cells were then washed with PBS and fixed with 4% paraformaldehyde (10 min). The fixed cells were then blocked with FBS followed by incubating with UEA lectin conjugated FITC overnight and imaged using fluorescent microscopy (Zeiss LSM510).

2.3.2. Human eNOS transfection

I have inoculated 1.2×10^5 BOECs/well into 6 well-plates, cultured for 24 h (about 50 – 60% confluence), and transfected with PVAX-eNOS, pMini-CMV-eNOS, pMini-EF1 α -eNOS with jetPRIMETM transfection reagent (200 μ L transfection mix into each well) as per manufacturer's instructions (1 μ g DNA, 200 μ L buffer, and 3 μ L of jetPRIMETM, Polyplus Transfection, New York, NY). The media was replaced 4 h after transfection

and samples were analyzed 24 h – 72 h later.

2.3.3. Minicircle eNOS

Parental plasmid pMini-EF1 α -eNOS was constructed for producing minicircle DNA. To construct pMini-EF1 α -eNOS, I have excised eNOS cDNA from pMini-CMV-eNOS constructed by technician in the lab by digesting at ScaI and EcoRI sites. The eNOS expression cassette was then ligated to linear pMini-EF1 α (System Biosciences, Palo Alto) at 4 °C overnight. Transformation was performed using ZYCY10P3S2T competent cells (System Biosciences, Palo Alto) and selected on a kanamycin (50 μ g/mL) containing LB agar plate. Positive clones were verified by restriction enzyme digestion and analyzed using gel electrophoresis.

2.3.4. Induction and Purification of Minicircle DNA

The induction of minicircle eNOS was performed as suggested by manufacture. On day 1, I grew cells from a mini-eNOS plasmid in 5 mL LB containing kanamycin (37 °C, 250 rpm). After 8 hours culture, I have combined 100 μ L of the culture with 400 mL of fresh TB (terrific broth) containing kanamycin and cultured at 37 °C, with shaking at 250 rpm. The value OD600 of the overnight culture should maintain 4 to 5 while pH is between 6 to 8 after 16 hours of culture. I have then combined minicircle induction mix (400 mL LB, 16 mL 1N NaOH and 400 μ L 20% L-arabinose) with the overnight culture and incubated it at 30 °C with shaking at 250 rpm for 5 hours. Bacteria were then pelleted and DNA was extracted. The induction of minicircle eNOS was verified by DNA gel with the

appearance of eNOS (4.1 kb) and minicircle backbone (1.6 kb) after digestion at ScaI and EcoRI sites.

2.3.5. Western blot

For eNOS detection, I have lifted and pelleted BOECs and proteins were extracted using cold RIPA buffer (Sigma-Aldrich, Oakville, Ontario, Canada). I have used DC protein assay (Bio-Rad, Mississauga, Ontario, Canada) to determine protein concentrations. I have mixed 20 µg of total protein with LDS sample buffer, heated to 70°C (10 min), and the reducing agent (DTT) was then added. Samples were run on Nupage 3-8% Tris-Acetate gels (Life Technologies, Burlington, Ontario, Canada), dry transferred with the iBlot system (Life Technologies, Burlington, Ontario, Canada) and the membranes were divided, blocked with 5% skim milk, and incubated with either a primary antibody for eNOS (Cat#: 07-520, Millipore, Billerica, MA) or β-actin (Cat#: A5441, Sigma-Aldrich, Oakville, Ontario, Canada) for 4°C overnight. The IRDye 800CW (Cat#: 925-32211) and IRDye 680RD (Cat#: 926-68070) secondary antibodies (LI-COR Bioscience, Lincoln, NE) were used for visualization on an Odyssey Imaging System (LI-COR Bioscience, Lincoln, NE).

2.3.6. Adhesion Assay

Osteopontin (R&D Systems, Minneapolis, MN) was prepared in PBS and surfaces incubated overnight at 4°C, blocked with 10% bovine serum albumin (BSA, Cell Signaling Technologies, Danvers, MA) for 1 h at 37°C and washed with PBS. I have inoculated 2.5×10^3 BOECs transfected with eNOS or non-transfected cells into 24-well

plates and incubated at 37°C for 4 h, wells were then washed with PBS, fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. Stained cells were visualized under 20X and 40X objectives. All cells were imaged with phase contrast microscopy, and numbers and morphology were computer analyzed (Image J 1.46).

2.3.7. Migration assays

For eNOS transfection study, I have plated cells transfected with eNOS or empty vector on the upper chamber of the insert containing 0.5% FBS EBM-2 while in the bottom chamber VEGF (50 ng/mL) or SDF-1 α (100 ng/mL) were used as chemoattractants. I have inoculated 3×10^5 BOECs to the upper compartment and cultured for 4 h, fixed with 4% paraformaldehyde (10 min), and stained with DAPI (5 min). Membranes were then placed on slides and migrating cells were counted by fluorescent microscopy (Zeiss LSM510).

2.3.8. Cytoskeletal and focal contact staining of adherent cells

To detect cytoskeleton and focal adhesion contacts, I have inoculated 1×10^4 cells/well into 12 well-plate containing 18 mm cover slips (either coated or non-coated). After 4 hours of culture, adherent cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton-X in PBS for 3 min, and blocked with 5% FBS PBS for 1 hour at RT. Focal contact sites were probed with a monoclonal anti-vinculin antibody (Sigma Aldrich, Oakville, ON, Canada) overnight at 4 °C, and 1 hour incubation with a secondary antibody conjugated with Alexa Fluor 488 followed by staining with rhodamine phalloidin (20 min, Life Technologies, Burlington, Ontario, Canada) and

DAPI (5 min). All samples were washed (PBS), mounted, and assessed with confocal microscopy (Olympus Fluoview FV1000).

2.3.9. Statistical analysis

Comparison between groups was performed with Student's *t* test or for multi-group comparisons by analysis of variance (ANOVA) followed by Tukey's post hoc test. All analysis was performed with GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA); values shown graphically are mean \pm standard error.

2.4. Results

2.4.1. Characterization of MACs and BOECs

I have found the average number of white blood cells (WBC) obtained from an apheresis sample was 1.1×10^{10} , with 13.5% of these cells being monocytes. Upon initial plating leukapheresis product into fibronectin-coated flask, cells start to display round morphology and after 5-7 days of culture, spindle shaped cell population emerges. This early population was non-proliferative and heterogeneous in morphology at day 7. This population is defined as "MACs" (Fig. 4A). A second population of cells emerged, typically after day 14 (Fig. 4B); these cells spread on the substrate and proliferated rapidly. With time in culture this second population took on a homogenous cobblestone appearance (Fig. 4C) and displayed contact inhibited growth characteristics. I found that these BOECs were similar in antigen profiles to HUVECs being positive for CD144,

CD309, vWF, UEA lectin, acLDL uptake and CD31, and negative for CD14, CD34, and CD45 (Figs. 4D and E). Mini-array data demonstrated 95% of mRNA expression is similarly expressed in BOECs compared to classic endothelial cells.

2.4.2. eNOS transfection

When BOECs were transfected with the jetPRIMETM-eNOS construct, I have found that eNOS levels rose to be comparable to or greater than those in HUVECs. Non-transfected BOECs expressed much lower levels of endogenous eNOS than did HUVECs (Figs. 5A and B). Cell loss was also seen with jetPRIMETM transfection yet the cell growth was restored from day 2 to 5 after transfection, with the transfected population undergoing a 2.6-fold increase during this period (Fig. 5C).

Induction of Minicircle eNOS was confirmed in gel electrophoresis after enzyme digestion (Fig. 6A). I found that production of minicircle DNA encoding eNOS was showed up to 2.6 times more eNOS expression compared to conventional plasmid (PVAX-eNOS) and pMini-EF1 α -eNOS in BOECs at 24 hours after transfection. At 72 hours, pMini-CMV-eNOS showed significantly higher (5.7 ± 0.8 , 1.8 ± 0.6 and 2.7 ± 0.7 folds for pMini-CMV-eNOS, PVAX-eNOS and pMini-EF1 α -eNOS) eNOS expression than conventional plasmid eNOS and pMini-EF1 α -eNOS (Figs. 6B, C and D).

2.4.3. eNOS transfection on cell migration and angiogenesis

With the induction of chemoattractants (VEGF and SDF-1 α) in the bottom chamber for 4 hours, I have observed greater number of migrated eNOS-transfected BOECs at the

bottom part of the insert compared to empty vector-transfected BOECs (55.5 ± 14.3 v.s. 99.8 ± 16.1 cells/field for empty vector and eNOS transfected cells) (Fig. 7A).

For angiogenesis analysis, BOECs after transfection were plated on top of prepared growth factor reduced Matrigel. Network formation was visualized under microscope at 8 hours. I have observed greater tube length and higher number of nodes (0.59 ± 0.20 vs. 0.91 ± 0.16 % of total pixels (tube length), and 3.67 ± 2.19 vs. 5.67 ± 1.67 nodes/field (nodes) for empty vector and eNOS transfected cells respectively) in eNOS transfected BOECs compared to empty vector-transfected cells (Fig. 7B).

2.4.4. Adhesion and spreading of BOECs after eNOS transfection

Previous graduate student Wafa Altalhi has studied the effect of OPN coating on BOECs adhesion and migration and she has found that OPN enhanced BOECs substrate adhesion and spreading in a dose-dependent manner (adherent cells increased from 39.5 ± 3.6 , 73.6 ± 1.7 , 88.4 ± 1.7 , to 111.9 ± 6 cells/field for 0, 1, 10, and 100 nM of OPN concentrations, respectively) (Figs. 8A and B). In addition the proportion of spread cells, defined as a cell area greater than $1500 \mu\text{m}^2$, in each adherent population was similarly increased with OPN ($38.1 \pm 4.5\%$, $58.7 \pm 0.9\%$, $66.1 \pm 2.5\%$, and $80.2 \pm 3.6\%$ for 0 nM, 1 nM, 10 nM, and 100 nM OPN coatings, respectively) (Fig. 8C). She had also demonstrated BOECs migration was significantly higher in response to haptotactic OPN than to chemotactic OPN (136 ± 20.3 cells/area vs. 53 ± 3.7 cells/area for haptotaxis and chemotaxis, respectively; $p < 0.01$, OPN (1 $\mu\text{g/mL}$)).

I have then taken on this project and found even a modest 1 nM OPN coating markedly increased the average cell area from $1264 \pm 245 \mu\text{m}^2$ (uncoated) to $2548 \pm 433 \mu\text{m}^2$ (coated; $p < 0.05$) (Fig. 8D). I have further studied the effect of OPN on eNOS transfected BOECs adhesion and spreading. I have found that eNOS transfection markedly increased cell adhesion to tissue culture plastic (39.6 ± 1.7 vs. 10.6 ± 1.1 and cells/field for eNOS-transfected vs. control cells, respectively; $p < 0.001$) and was enhanced further by the presence of OPN (16 ± 0.9 and 49.4 ± 2.4 cells/field for non-transfected cells and eNOS-transfected cells, respectively; $p < 0.001$) (Fig. 9A). Similarly, the percentage of cells defined as spread increased with eNOS transfection ($30.6 \pm 2.8\%$ and $84.7 \pm 3.5\%$ non-transfected vs. eNOS-transfected EPCs on plastic coatings, and $34.0 \pm 3.3\%$ vs. $92.1 \pm 3.9\%$ for non-transfected and eNOS-transfected cells on 1 nM OPN coatings, respectively) (Fig. 9B). For spread cells the combination of eNOS transfection and OPN coating increased average cell area ($2186.8 \mu\text{m}^2$ vs. $4991.2 \mu\text{m}^2$ for non-transfected cells on 0 nM OPN coating vs. eNOS-transfected cells on 1 nM OPN coating). Both eNOS transfection and OPN coatings had profound effects on the cell cytoskeleton (F-actin) and focal adhesion site distribution (Fig. 9C) with OPN producing dense focal adhesion sites on the cell periphery, whereas the combination of eNOS transfection and OPN produced more focal adhesions distributed throughout the cell.

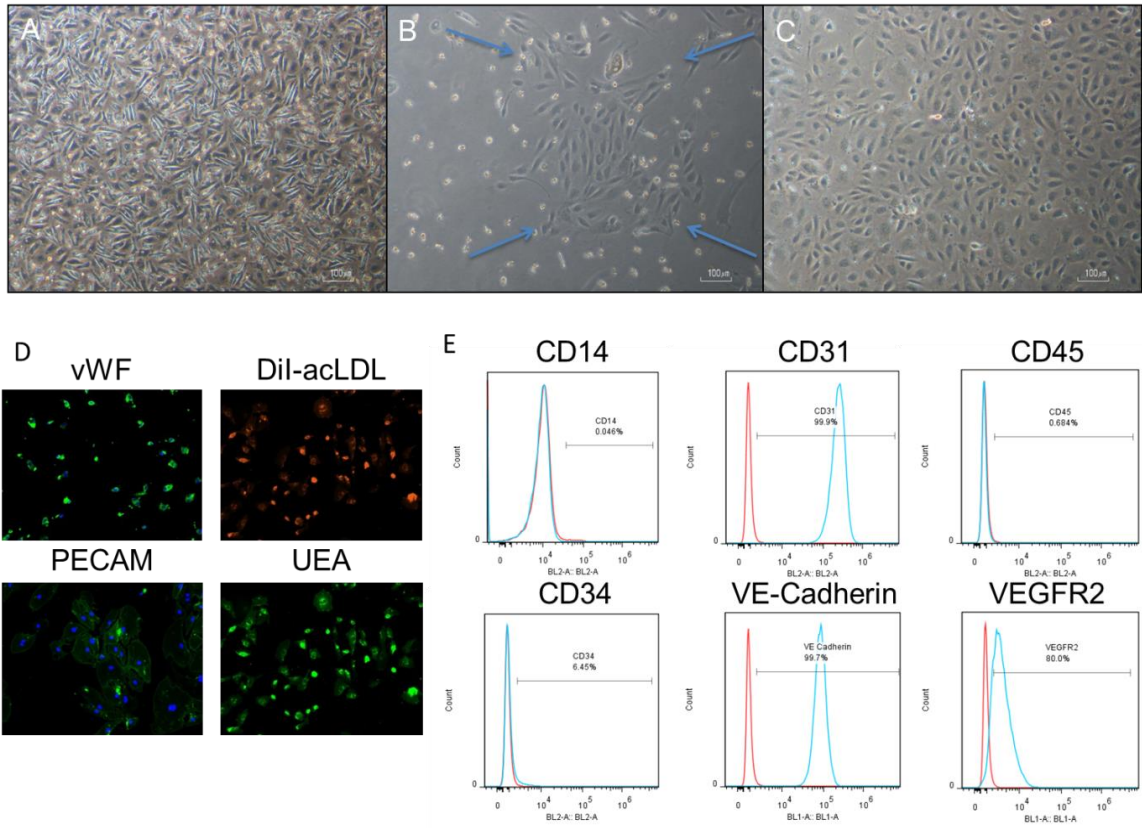


Figure 4: Morphological progression of peripheral blood-derived MACs (day 5 and 21; A and B) and third passage BOECs (C). Fluorescent images of stained BOECs are to test expression of vWF, PECAM, acLDL uptake and UEA lectin (D). Cells were analyzed for the surface markers including CD14, CD45, CD31, CD34, VE-Cadherin and VEGFR2 by flow cytometry (E).

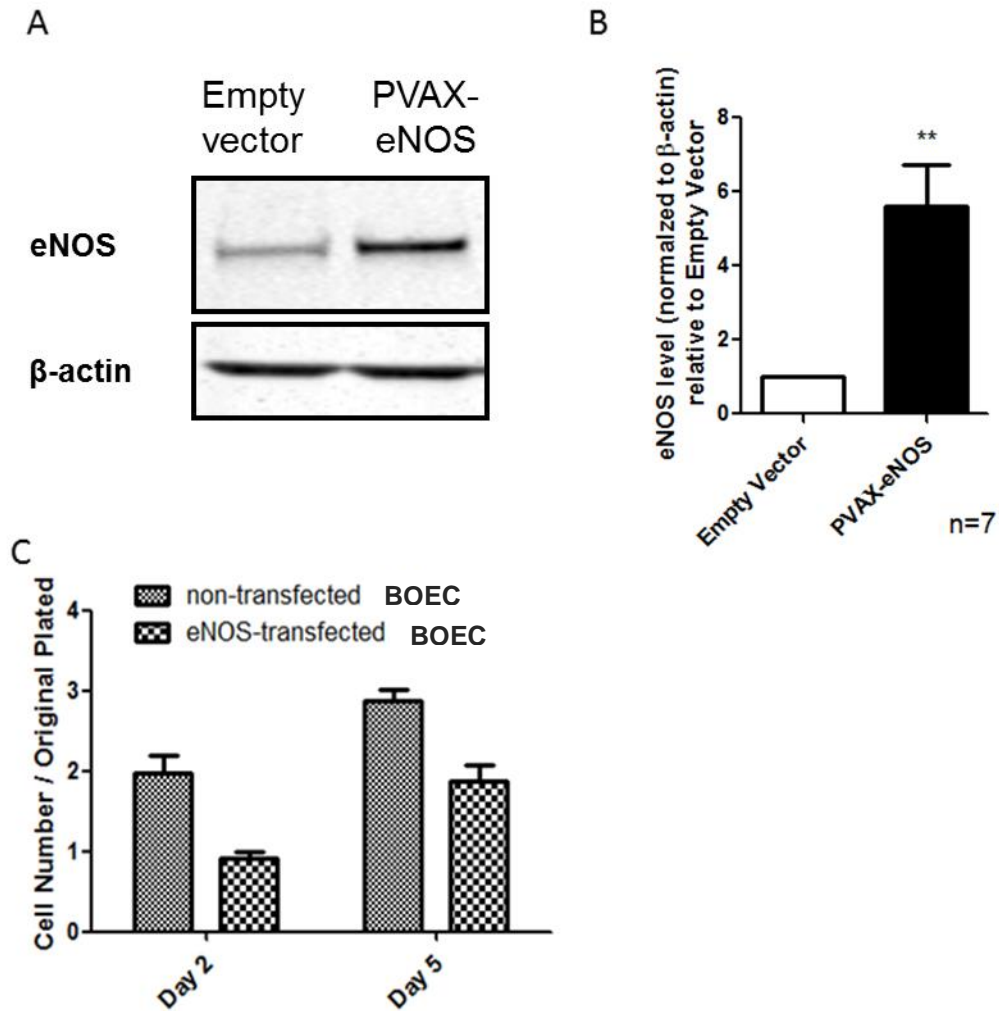


Figure 5: Western blot analysis of jetPRIME-based transfection of BOECs. BOECs null transfected (empty vector), and BOECs eNOS transfected (A) analyzed by densitometry with bands normalized to β -actin and non-transfected BOECs level (B). Adherent cell number was quantified on day 2 and day 5. Data presented as mean \pm SEM. n=3 (C). ** indicates $p < 0.01$. Reprinted from [201] with permission from Elsevier.

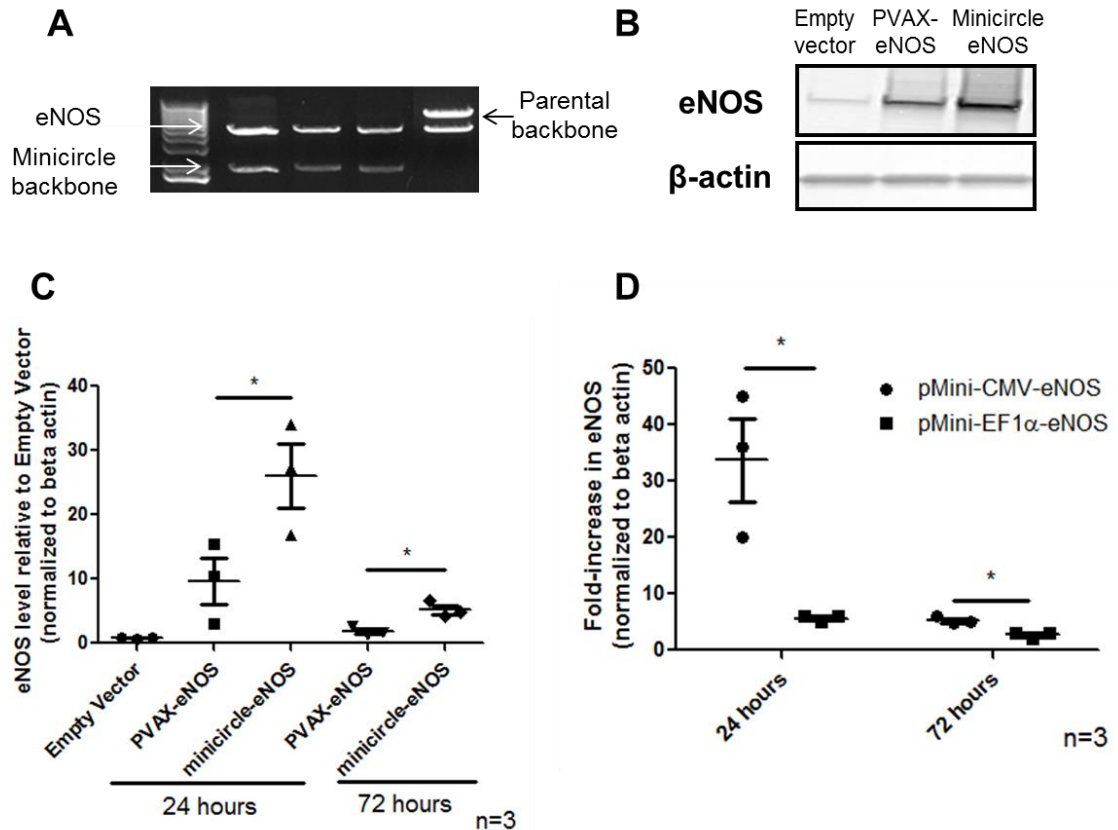


Figure 6: Analysis of the production of minicircle DNA (pMini-CMV-eNOS) under designed culture conditions. After induction, parental minicircle DNA was digested with EcoRI and ScaI. After digestion, backbone plasmid was linearized (10kb), pMini-CMV-eNOS was cut into two linear fragments: eNOS (4.1kb) and minicircle backbone (1.6kb) (A). Western blots analysis of BOECs transfected with empty vector, PVAX-eNOS and pMini-CMV-eNOS (B). Densitometry results showed eNOS level normalized to β -actin of 24 hours and 72 hours culture (C). Densitometry analysis presented the comparison of different promoters (CMV and EF1 α) in minicircle transfection (D). Data are presented as mean \pm SEM, * indicates $p < 0.05$. n=3.

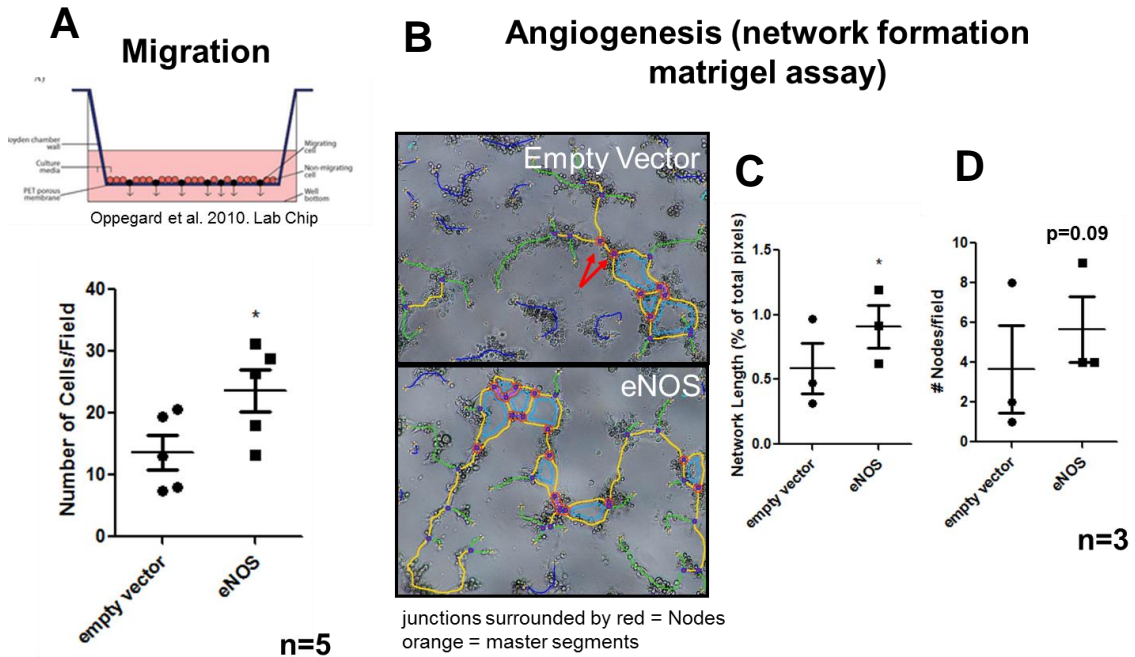


Figure 7: the effect of transient transfection of eNOS on BOEC migration and angiogenesis. Cells after transfection were harvested and cultured for boyden chamber assay for 4 hours. The number of migrated BOECs was identified by DAPI staining (A). Cells transfected with either empty vector or eNOS plasmid were cultured on matrigel for 8 hours followed by microscopical detection. Nodes were indicated by junctions surrounded by red and tube was indicated by yellow lines (B). The length of network and number of nodes were counted in different conditions (C, D). Data are presented as mean \pm SEM, * indicates $p < 0.05$.

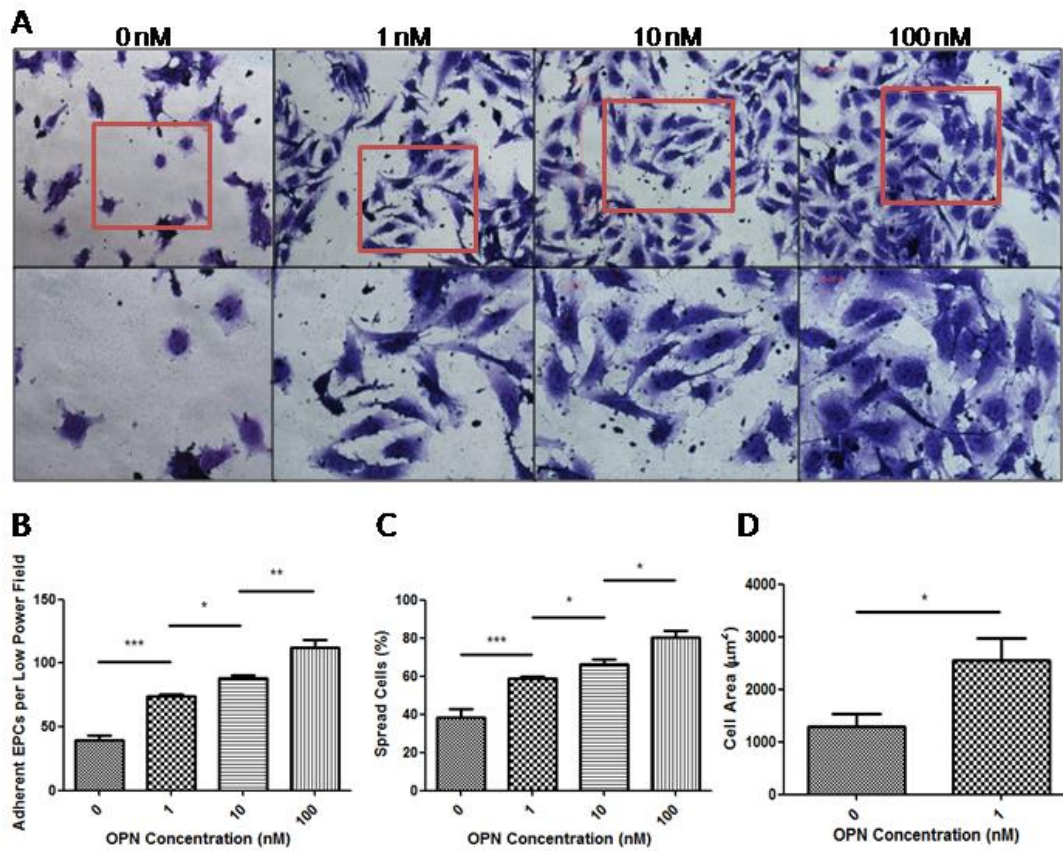


Figure 8: Adhesion of BOECs to different concentrations of immobilized OPN (0 nM, 1 nM, 10 nM and 100 nM), paired photomicrographs (A) of crystal violet stained cells under low (upper) and high power (lower); red box indicated region of high power field. The number of adherent EPCs were counted under low power field (B), and the cell area calculated by image analysis; the percentage of spread cells defined as cells exceeding $1500 \mu\text{m}^2$ (C) and cell area (D) are plotted. Data presented as mean \pm SEM, *, **, ***

indicate $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively, $n=3$. Reprinted from [201] with permission from Elsevier.

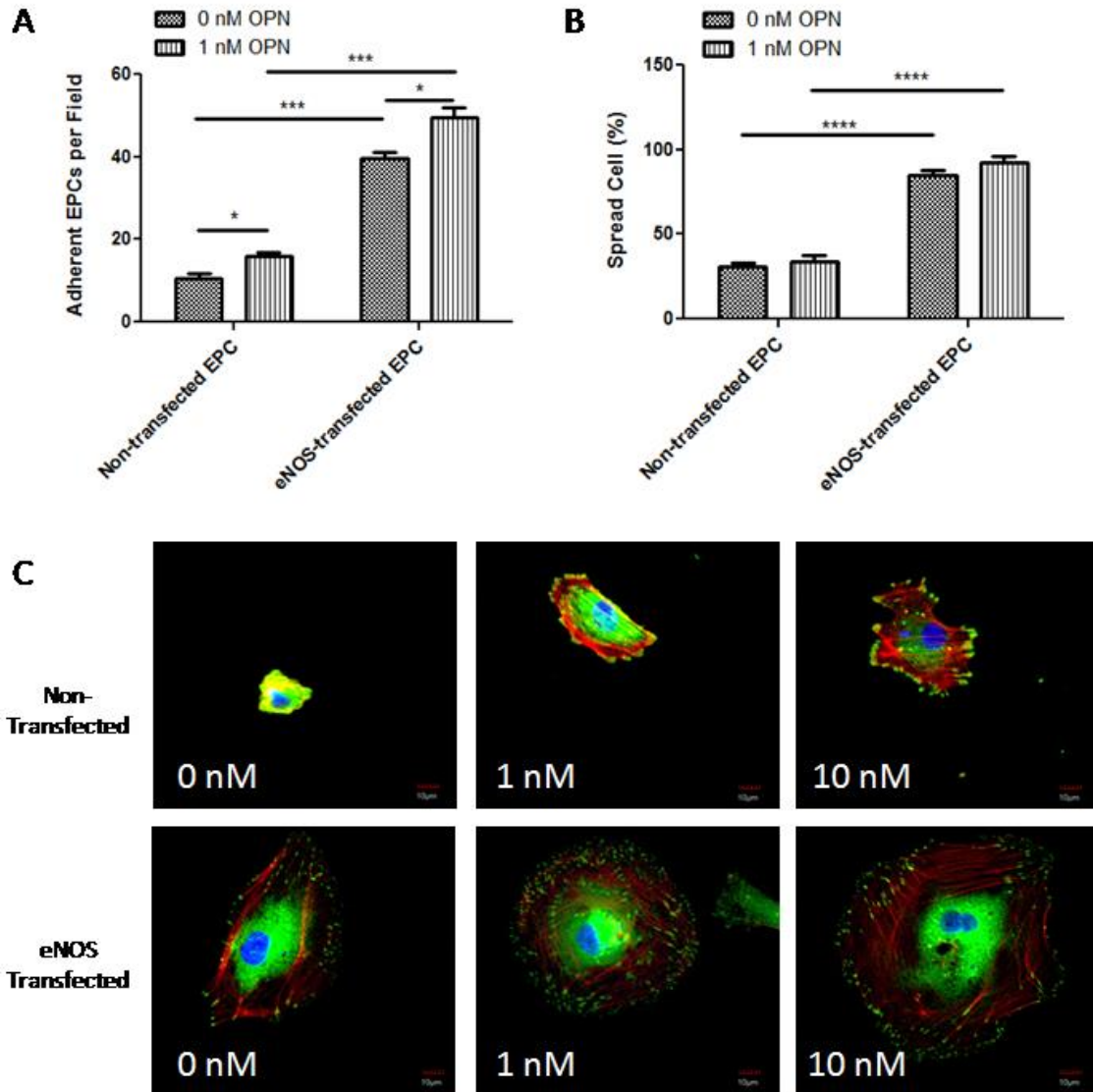


Figure 9: jetPRIME-based plasmid eNOS transfection enhanced BOEC adhesion (A) and spreading (B) on both OPN coated (1 nM) and uncoated surfaces. Data presented as mean \pm SEM, $n=4$. *, **, ***, **** indicate $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively. The cell cytoskeletal network and focal contacts were assessed by F-actin

staining (red), vinculin (green), and nuclei counter-stained with DAPI (blue). Transfected or non-transfected EPCs were imaged on substrates coated with OPN (0 nM, 1 nM, and 10 nM) (C). Reprinted from [201] with permission from Elsevier.

2.5. Discussion

In this study, I describe a minimally invasive protocol, using xenogene free reagents, to readily derive up to 10^9 human BOECs, from a single donor apheresis product. These BOECs were phenotypically identical to human endothelium in terms of morphology, growth, kinetics, and surface marker expression, and as such represent an ideal source of autologous endothelium for seeding onto cardiovascular biomaterials. Interestingly, although displaying 95% of mRNA homology to classic endothelial cells, the eNOS basal expression of BOECs was markedly reduced. Their angiogenic potential can be enhanced by eNOS transient transfection. When combined with OPN-modified surfaces, a potent extracellular matrix protein, eNOS transfection can synergistically enhance cell adhesion and spreading. Taken together, these cell and surface modifications provide a method to efficiently cover material surfaces with a highly functional and autologous endothelial layer.

EPCs have been previously isolated from adipose and bone marrow [215,216]; however, these collection procedures are invasive and subsequent cell processing more complex. Leukapheresis is an established and well-tolerated clinical procedure that leads to fewer complications [217,218]. A number of groups have derived MACs from human

leukapheresis products [219,220]. Although MACs may induce angiogenesis, they are distinct from mature endothelium and phenotypically resemble monocytes [81], thus they would be a poor choice for the establishment of endothelial monolayers in culture. In contrast, others and I have shown that BOECs show a cobblestone morphology similar to ECs, have a high proliferative capacity [98], and also exhibit antigen profiles (i.e., CD31, 133, 144, 15, 146, KDR, Ac-LDL, and vWF) similar to mature ECs [221]. They closely resemble HUVECs in culture and may be a promising therapeutic option for in-vitro re-endothelialization [78]. To my knowledge, I am the first to report the generation of BOECs from large-volume apheresis products although many other groups have demonstrated obtaining MACs or EC colonies; however, most of these studies used G-CSF pre-treatment in the donors [220,222,223]. Because G-CSF potently stimulates WBC production and depresses the angiogenic potency of EPCs, it may not be tolerated well in severely ill cardiovascular patients [224,225]. In my study, I have isolated and expanded BOECs from apheresis products without pre-treating the donors with G-CSF. This was performed under xenogene free conditions (EBM-2 media containing recombinant human growth factors, and supplemented with 20% human serum on human fibronectin-coated flasks) that allowed for more than a 200-fold expansion. Previous studies have utilized FBS for BOEC expansions [226] yet the use of animal products would be less desirable for the development of clinical materials.

OPN is a matricellular protein that plays an important role in both cell adhesion and cell signaling through interaction with cell surface integrins and CD44 [212,227]. Recent studies suggest that OPN stimulates the repair of denuded arterial surfaces in vivo [210]. The biological activity of OPN likely involves enhanced endothelial adhesion and

migration mediated by binding to $\beta 1$ subunit containing and $\alpha_v\beta_3$ integrins [211,212,214,228,229]. A recent study has indicated that OPN in solution inhibits adhesion, proliferation, and the potential for vasculogenesis of human umbilical cord-blood derived EPCs [230]. Wafa Altalhi and I found similar results on our adult-derived BOECs when OPN in solution was used in chemotactic assays. However, we found that immobilized OPN induced 3 times more BOEC migration than chemotactic migration to OPN in solution. Additionally, immobilized OPN increased cell adhesion in a dose- and time- dependent manner. These results compared to those of Yu et al. highlight the biologically distinct responses to soluble versus immobilized OPN [230]. Immobilizing OPN on biomaterial surfaces may promote endothelialization, and at the same time mitigate many of the potential negative attributes of this protein.

Endothelial NOS, regulates the production of NO, mediates a variety of angioprotective functions (including neovascularization), blood vessel tone, and is anti-thrombotic preventing platelet adhesion [166,206]. As such eNOS is an ideal candidate gene for restoring endothelial functional activity for cells seeded onto blood-contacting surfaces. To examine the role of eNOS expression on BOEC functional activity I performed transfection studies with minicircle DNA. I have first demonstrated the effectiveness of jetPRIME™ for plasmid-based eNOS transfection of BOECs. Although it initially reduced cell survival, this effect was transient and the cells recovered and thrived. Minicircle DNA is devoid of bacterial backbone that mediates the spread of transcriptionally repressive chromatin deposition [231]. It has been tested in multiple gene transfection studies such as VEGF [209] and hypoxia-inducible factor 1 α (HIF1 α) [208] and was demonstrated to achieve sustained and enhanced transgene expression

compared to plasmid-based transfection. In line with previous findings, I have shown here that transfection with minicircle DNA resulted in a significantly higher level of eNOS expression compared to that of conventional plasmid at both 24 and 72 hours. pMini-CMV-eNOS also produced more sustained eNOS expression (3.1 ± 0.7 fold increase at 72 hours). I have also tested two different promoters CMV and EF1 α to drive eNOS expression. After 24 and 72 hours, pMini-CMV-eNOS achieved significantly higher level of eNOS expression compared to pMini-EF1 α -eNOS. Similar to my study, Liu and colleagues have stably transduced MACs and BOECs using vectors with CMV or EF1 α promoters and demonstrated that transgene expression in both in vitro and in vivo studies was higher under the control of CMV promoter compared to EF1 α promoter [232] although another study proved the opposite in mesenchymal stem cells (MSCs) [233], suggesting that the efficacy of promoter is cell-dependent. Furthermore, I found the angiogenic potential of BOECs was enhanced after eNOS transfection in vitro. When combined with OPN coated surfaces, eNOS-transfected BOECs displayed markedly enhanced substrate adhesion and spreading. Although eNOS expression reduced within days after transfection, it is likely that this transient response would be sufficient to establish the cells on a vascular surface and mitigate initial post-implantation thrombotic responses. Bor-Kucukatay et al. have reported that nitric oxide can modify the cytoskeleton of cells [234]. I observed that the cytoskeleton of the BOECs was altered and more focal adhesion sites present with transfection. Enhanced cell adhesion may be related to increased cell deformability and spreading, leading to more cell substrate contact points. Increases in cell adhesion and area may stimulate the rapid establishment

of an endothelial monolayer. Thus eNOS-transfected BOECs may be ideal for endothelial regeneration.

**CHAPTER 3: THE REGULATION OF ENOS
BY EXTRACELLULAR MATRIX IN BOECS**

3.1. Introduction

A readily available autologous and functional endothelial population is critical for endothelialisation of blood contacting biomaterial surfaces but hard to achieve. Our previous microarray data has shown that BOECs, cultured from blood mononuclear cells, display 95% homology of mRNA expression to that in mature endothelial cells (i.e. HUVECs and HMVECs) [201]. However, my Western Blot data in Chapter 2 demonstrates that unlike mature endothelium, eNOS expression is limited in BOECs. These findings suggest eNOS is regulated differently in BOECs compared to mature endothelial cells. EPCs originate from bone marrow and circulate freely in the blood whereas mature endothelium remains attached to the vessel wall [221]. The cellular microenvironment including ECM components, cell-cell interactions, oxygen tension etc. may be responsible for the small but significant phenotypic differences between BOECs and mature endothelial cells.

ECM is a collection of extracellular molecules that provides structural support and regulates cell adhesion and cell signaling. Endothelial cells are lining the inner surface of the vascular lumen and in direct contact with basement membrane which is composed of various ECM components. ECM remodeling has been shown to directly or indirectly result in cardiovascular disease progression ([235]; reviewed in [190,191] and reviewed in [195]). For example, Passerini and colleagues have demonstrated that there are about 2000 differentially expressed genes between ECs harvested from atherogenetic and healthy regions, respectively and the main cause of this difference is through changes of cell-ECM interactions which involve molecules such as integrin, c-Src/extracellular signal-related kinase $\frac{1}{2}$ (ERK $\frac{1}{2}$), focal adhesion kinase, and actin cytoskeleton ([178]

[193] [194] [192] and reviewed in [195] [190]). Further studies have specifically demonstrated the tight correlation of cell-ECM interactions and eNOS signaling [180-182]. Compared to normal basal membrane ECM components (e.g. Col. IV), abnormal ECM protein (e.g. Col. I) in vessel walls could be responsible for the decreased NO production through ILK dependent pathways [180]. FN and LAMA which are the main ECM components in the vascular basement membrane, have both been shown to negatively regulate eNOS expression and activity possibly through p38 MAPK dependent pathway in endothelial cells [181,182]. Thus, there might be a negative regulation of eNOS expression/activity by cells binding to ECM substrates.

Endothelium is primarily responsible for the synthesis and deposition of basement membrane ECM components which in turn regulate many biological functions of ECs (reviewed in [236]). One recent study has compared the deposition of multiple ECM components such as Col. IV, FN, and LAMA in umbilical cord blood BOECs, umbilical vein ECs, and umbilical artery ECs and demonstrated that an organized weblike structure has been observed in BOECs culture whereas other ECs only express these ECM proteins intracellularly. They have further shown that BOECs highly deposit Col. IV and LAMA compared to other cells [237]. Since ECM substrates may negatively regulate eNOS signaling in endothelial cells, I hypothesized that EC deposited ECM downregulates expression and activity levels of eNOS. In this study I will explore the biological mechanisms of eNOS regulation by ECM proteins in BOECs.

3.2. Specific Aims

- To quantitatively measure the differential expression of key matrix proteins in BOECs, HUVECs and HAECs.
- To determine differential regulation by key matrix proteins on eNOS mRNA and protein expressions in BOECs and HUVECs.
- To demonstrate the negative regulation of eNOS by endogenously deposited Col. I in BOECs culture.

3.3. Methods and Materials

3.3.1. Cell Culture

Upon isolation, BOECs were cultured on fibronectin (Roche Applied Sciences, Indianapolis, IN) coated flasks in supplemented Endothelial Basal Medium-2 (EBM-2) (Clonetics, Lonza, Walkersville, MD) containing 20% human serum for the first passage. BOECs were then cultured in polystyrene-coated plates with the same culture medium. HUVECs and HAECs were cultured in the same culture medium on polystyrene-coated plates for all experiments. Cells were passaged once reach 80% confluency and passage 3 – 6 cells were used throughout the experiments.

3.3.2. Western blot

For eNOS protein detection, Nupage 3-8% Tris-Acetate gels were used as previously described [2]. To extract whole cell protein, I have lysed BOECs and collected by cell scraper in cold RIPA buffer supplemented with protease inhibitor (Roche Applied Sciences, Indianapolis, IN), sodium fluoride and sodium orthovanadate (Sigma Aldrich,

Oakville, ON, Canada). Protein concentrations were determined using lowry assay (Bio-Rad) and 30 – 40 µg of total protein mixed with LDS sample buffer, heated to 70 °C (10min), and the reducing agent (DTT) was then added. After gel transfer, I have used 5% skim milk to block nitrocellulose membrane followed by incubation with following primary antibodies: eNOS (Cat#: 07-520, Millipore, Billerica, MA) or β-actin (Cat#: A5441, Sigma-Aldrich, Oakville, Ontario, Canada) as control at 4 °C overnight. The IRDye 800CW (Cat#: 925-32211) and IRDye 680RD (Cat#: 926-68070) secondary antibodies were used for visualization on an Odyssey Imaging System.

3.3.3. Quantitative Real-time reverse transcription-PCR

Total mRNA was extracted from different cells in indicated conditions by using RNA isolation system (Exiqon, Woburn, MA). I have used a cDNA synthesis kit (Life Technologies, Burlington, ON, Canada) to synthesize cDNAs. The mRNA levels of eNOS and Col. 1 was analyzed using Taqman primers (Life Technologies) (NOS3: Hs01574659_m1; Col1A1: Hs00164004_m1; FN1: Hs00365052_m1; Col4A1: Hs00266237_m1; LAMA: Hs00189308_m1) and Real-Time PCR system (Bio-Rad). Relative changes in mRNA expression of target genes were determined using $\Delta\Delta C_t$ method normalized to 18S.

3.3.4. Immunocytochemistry

To detect cytoskeleton and focal contact contacts, I have inoculated 1×10^4 cells/well into 12 well-plate containing 18 mm cover slips (either coated or non-coated). After 3 days of culture, adherent cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton-X in PBS for 3 min, and blocked with 5% FBS PBS for

1 hour at RT. For detection of extracellular matrix proteins, cells were incubated with primary antibodies as following: Col. I (Cat#: ab34710), FN (Cat#: ab2413) and laminin (LAMA)(Cat#: ab11575) (all from Abcam, Toronto, ON, Canada) overnight at 4 °C, respectively. All samples were washed (PBS), mounted, and assessed with confocal microscopy (Olympus Fluoview FV1000).

3.3.5. Decellularization

I have seeded 1.5×10^4 cells/well (BOECs and HUVECs) onto 18 mm cover slip and cultured until 80 – 90% confluence. I have then removed culture medium and overlying cells were lysed by incubating with 50 mM NH_4OH buffer containing 0.5% Triton X for 5 min followed by PBS wash for 5 min. Immunocytochemistry was then performed to verify the collagen I content left on cover slip.

3.3.6. Hydroxyproline content

I have cultured about 5×10^3 cells/cm² (BOECs and HUVECs) for 3 days in T75 flasks and 80% confluence can be reached. After decellularization, the remaining matrix were washed with cold PBS and collected in lysis buffer and used for hydroxyproline measurement. The samples (200 μL) were solubilized with the same volume of 12N HCl and heating to 120°C for 3 hours and then 10 μL of supernatant was transferred to a 96 well-plate. The acid was removed by evaporation (60 °C oven for 20 – 30 minutes). In each well, 100 μL of the chloramine T/Oxidation buffer mixture was added and incubate at RT for 5 minutes. Then add 100 μL of the diluted DMAB reagent and incubate for 90 minutes at 60 °C. A standard curve of 0, 0.1, 0.2, 0.4, 0.8 μg hydroxyproline was constructed to calibrate absorbance values by diluting the appropriate volume of a 0.1

mg/mL trans-4-hydroxy-L-proline solution (SIGMA) in assay buffer to a final volume of 100 μ L in a 15 mL tube. The concentration of hydroxyproline per cell was graphed.

3.3.7. ECM protein coating and cell culture

Same concentrations ($2 \mu\text{g}/\text{cm}^2$) of fibronectin (FN, R&D Systems, Minneapolis, MN), collagen I (Col. I, BD Biosciences, San Diego, CA) and Laminin (LAMA, Sigma Aldrich, Oakville, ON, Canada) were prepared in PBS. Surface coatings were performed by incubation with corresponding solutions at RT for 2 hours. After coating, surfaces were washed with 1XPBS three times before used for cell culture. Unused surfaces containing PBS were stored in 4 °C fridge.

To test the effect of ECM coating on cell culture, I have cultured 5×10^3 cells/ cm^2 HUVECs or BOECs on different ECM coated surfaces for 3 days in T25 flasks. At the end of culture, cells were harvested for western blot and qRT-PCR testing.

3.3.8. Silencing interference RNA (siRNA) Transfection

I have inoculated 1×10^5 BOECs/well into 6 well plates, cultured for 24 h followed by transfection with Col.I siRNA or scramble control (200 μ L transfection mix into each well) as per manufacturer's instructions (100 pmol siRNA, 200 μ L buffer, and 4 μ L of jetPRIMETM, Polyplus Transfection, New York, NY). The media was replaced 4 h after transfection and samples were analyzed at least 24 h later.

3.3.9. Statistical Analysis

Comparison between groups was performed with Student's t test or for multi-group comparisons by ANOVA followed by Tukey's post hoc test. All analysis was performed with GraphPad Prism 5.0; values shown graphically are mean \pm standard error.

3.4. Results

3.4.1. eNOS expression and activity

Upon cultured in the same medium, BOECs, HUVECs and HAECs presented similar homogenous cobble-stone morphology and rapid proliferation capacity with approximate doubling time of 36 hours. Western blot and qRT-PCR showed a markedly lower expression of eNOS in BOECs (0.34 ± 0.13 eNOS protein, $p < 0.05$; 0.29 ± 0.17 eNOS mRNA, $p < 0.05$) compared to HUVECs (Figs. 10A and B) while the eNOS mRNA expression in BOECs is further less compared that in HAECs (Fig. 10B). For mRNA stability study, different types of cells were cultured for 3 days followed by treatment with actinomycin D ($2.5 \mu\text{M}$), a mRNA synthesis inhibitor, for 24 hours and eNOS decay rate was found slower in HUVECs compared to BOECs (Fig. 10C). BOECs were also found to produce significantly lower nitrite (0.49 ± 0.18 nitrite, $p < 0.05$), which is an indicator of nitric oxide generation, compared to HUVECs (Fig. 10D).

3.4.2. eNOS regulation by ECM proteins

When grown on FN, Col. I and LAMA, I found significantly decreased eNOS protein in HUVECs (0.52 ± 0.08 fold change on FN, 0.49 ± 0.07 on Col. I and 0.51 ± 0.1 on LAMA) compared to cells on polystyrene (POLY) (Fig. 11A), these findings were associated with

decreased eNOS mRNA (0.57 ± 0.02 on FN, 0.68 ± 0.06 on Col. I and 0.58 ± 0.09 on LAMA) (Fig. 11B). However, in BOECs, eNOS expression was unchanged in any of the matrices (Figs. 11C and D). The reason might be that BOECs highly deposit ECM proteins and external provision is not necessary to stimulate intracellular signaling.

3.4.3. ECM expression

ECM substrate expressions were assessed in BOECs, HUVECs and HAECs. After 3 days culture, much more Col. I staining has been observed in BOECs compared to HUVECs, whereas the expression levels of FN and LAMA in these two cell types were similar (Fig. 12A). This finding has been further correlated with qRT-PCR data which shows that BOECs express markedly higher level of Col. I mRNA compared to HUVECs and HAECs (3.01 ± 1.48 (HAECs) and 41.67 ± 12.07 (BOECs) fold increases relative to HUVECs) (Fig. 12B) whereas FN and Col. IV are modestly different among these cells (Figs. 12C and E). Interestingly, the expression of laminin was markedly higher in HUVECs compared to other types of cells (Fig. 12D), however its absolute level was close to detection limit (cycle number was 38 for HUVEC) and has been disregarded.

3.4.4. Col. I expression

To further look into long term Col. I expression, BOECs and HUVECs were plated into culture for 7 days. Immuno fluorescent data showed that on days 3, 5, and 7, both the average (per cell) (109000 ± 3600 vs. 184000 ± 7100 (RAU/cell) for HUVECs and BOECs, respectively on day 3; 85000 ± 24000 vs. 159000 ± 8000 (RAU/cell) for HUVECs and BOECs, respectively on day 5; 89000 ± 18000 vs. 193000 ± 23000 (RAU/cell) for HUVECs and BOECs, respectively on day 7) and total Col. I staining

(1520000 ± 101000 vs. 2960000 ± 470000 RAU for HUVECs and BOECs, respectively on day 3; 1160000 ± 610000 vs. 4820000 ± 360000 RAU for HUVECs and BOECs, respectively on day 5; and 1090000 ± 28000 vs. 4140000 ± 810000 RAU for HUVECs and BOECs, respectively on day 7) in BOECs was significantly higher compared to that of HUVECs throughout entire culture period (Figs. 13B and C).

I have also observed that the collagen in BOECs can form mesh-like structures on days 3 and 5 whereas in HUVECs no organized structure was observed (Fig. 13A). Gene expression data further confirmed this finding that markedly reduced level of Col. I mRNA level were found in HUVEC compared to BOEC on days 3, 5, and 7 (62.69 ± 22.99 fold increase for day 3 BOECs compared to day 3 HUVECs; 1.28 ± 1.26 and 67.53 ± 36.36 fold increases for day 5 HUVECs and BOECs compared to day 3 HUVECs, respectively; 0.02 ± 0.01 and 65.36 ± 51.80 fold increases for day 7 HUVECs and BOECs compared to day 3 HUVECs, respectively)(Fig. 13D).

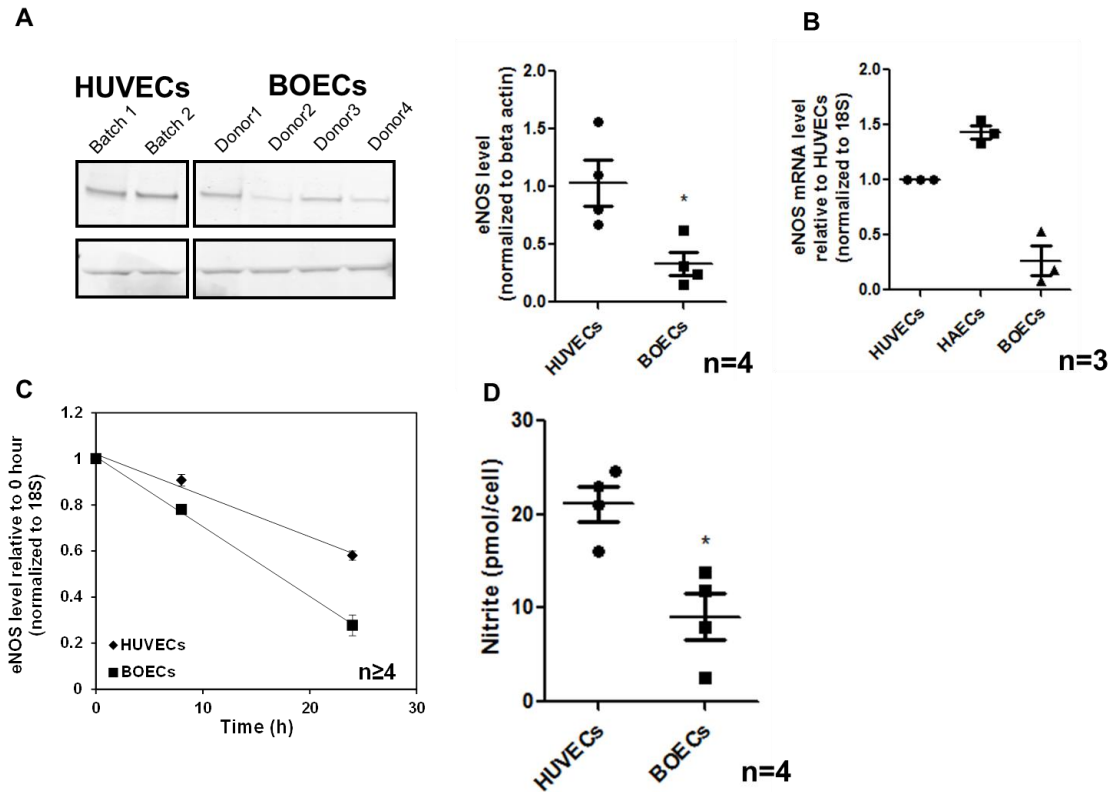


Figure 10: eNOS expression and activity in BOECs, HUVECs and HAECs. All cells were cultured in the same culture medium for 3 days followed by assessments for eNOS protein (Western blot) (A) and mRNA expression levels (RT-PCR) (B). To test eNOS mRNA stability, HUVECs and BOECs were cultured for 3 days followed by treatment with actinomycin D for 24 hours. At 0, 8 and 24 hours, mRNA samples were collected and measured for eNOS expression. The trend-line indicates eNOS degradation rate (C). The eNOS activity was indicated by nitrite level, which was measured in the supernatant in HUVEC and BOEC cultures on day 3. The average level of nitrite per cell was plotted (D). The n indicates donor number. * indicates $p < 0.05$.

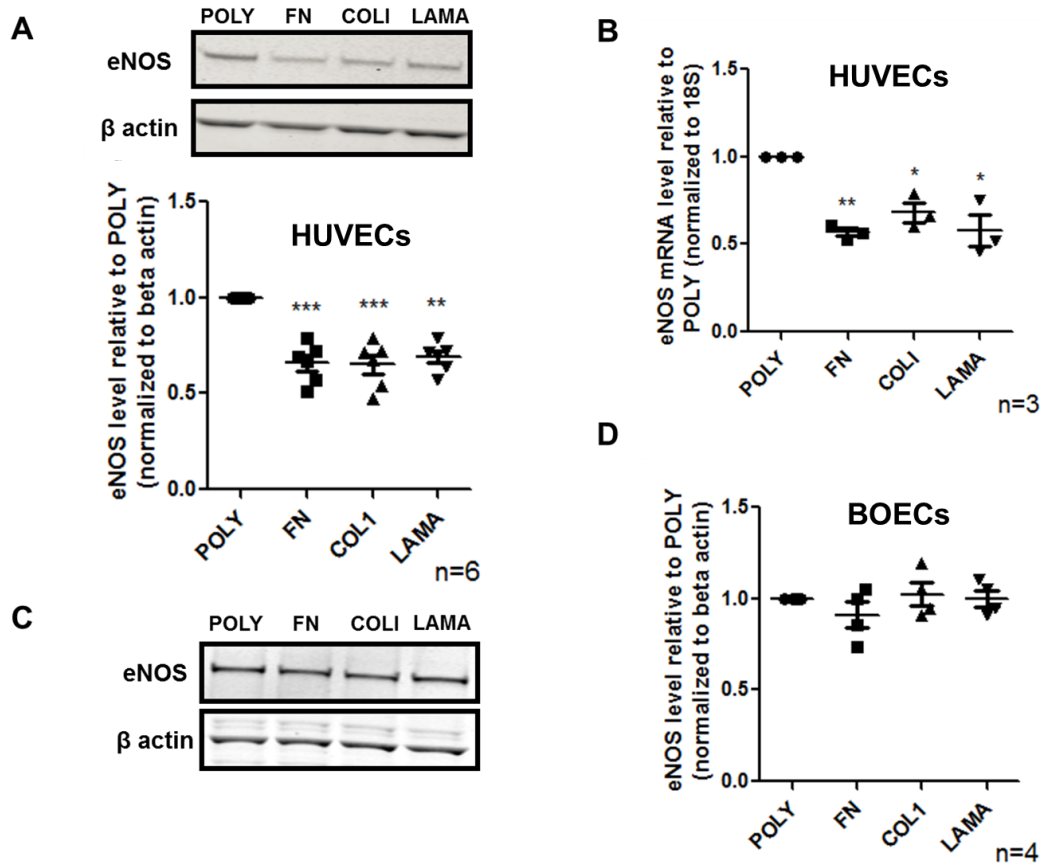


Figure 11: The regulation of eNOS by ECM proteins in HUVECs and BOECs. Cells were cultured on POLY, FN, Col. I and LAMA for three days followed by Western blot and qRT-PCR to assess eNOS protein (A for HUVECs, C and D for BOECs) and mRNA expression levels (B for HUVECs). n indicates experimental replicates. *, **, *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

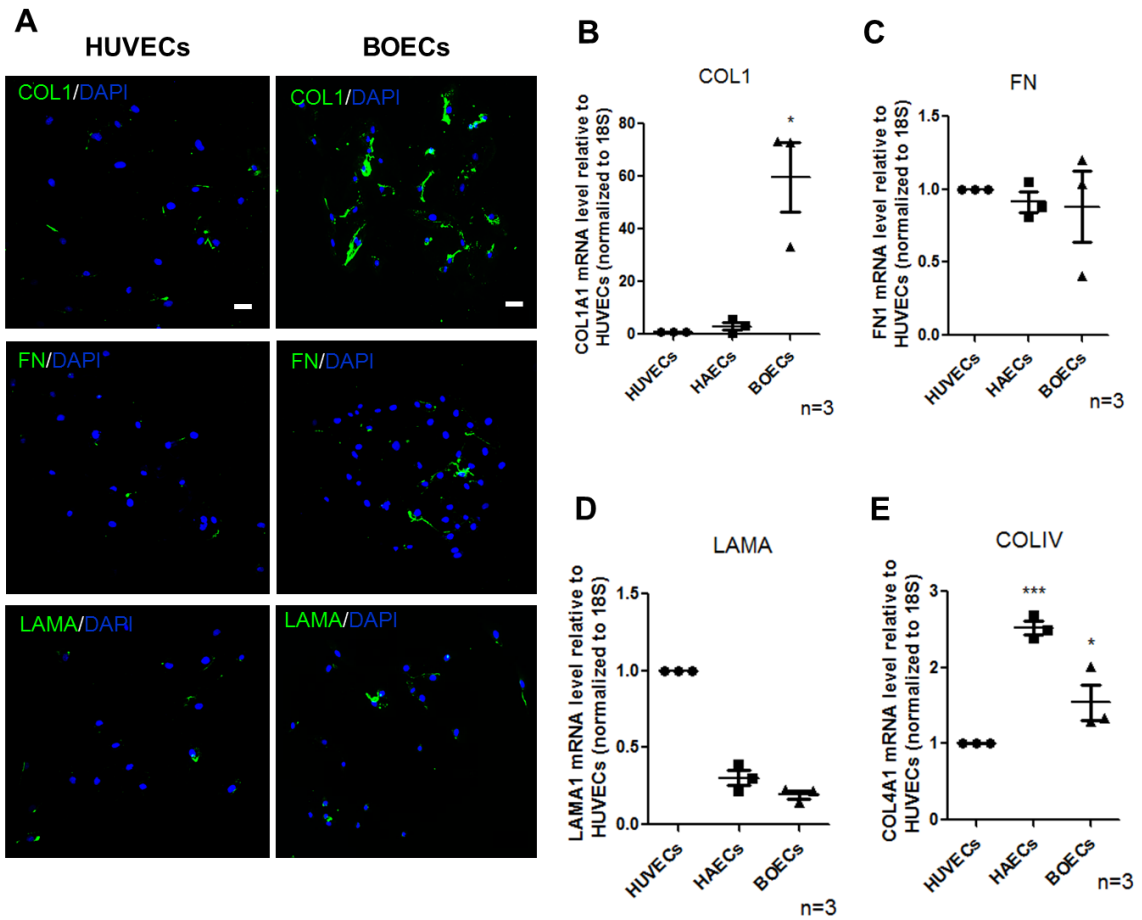


Figure 12: ECM molecule expressions in different endothelial cells. HUVECs, HAECs, and BOECs were cultured on polystyrene for 3 days and the protein (A) and mRNA (B – E) expressions of Col. I, FN, LAMA, and Col. IV were evaluated by immunofluorescent microscopy detection and qRT-PCR, respectively. n indicates experimental replicates. Scale bar indicates 20 μm . * and *** indicate $p < 0.05$ and $p < 0.001$, respectively.

3.4.5. Col. I Deposition

Following decellularization, ECM proteins on the plate of HUVECs and BOECs cultures were stained by Col. I antibody. I have noticed that BOECs highly deposit Col. I that assembles to an organized mesh-like structure whereas HUVECs only express few Col. I fibres which cannot form organized structure (Figs. 14A and B). Further results on hydroxyproline content, a major component of protein collagen, demonstrated that significantly higher hydroxyproline concentration (2.78 ± 1.02 fold increases) was obtained from BOECs in 4 independent donors compared to that in HUVECs (Fig. 14C).

3.4.6. eNOS regulation by Col. I siRNA

The expression of Col. I mRNA was significantly reduced in BOECs (0.31 ± 0.03 fold decrease compared to scramble siRNA transfected BOECs) (Fig. 15A) 24 hours after Col. I siRNA transfection. The expression level of Col. I assessed by immunostaining was found to be decreased in the Col. I siRNA transfected group compared to the scrambled vector transfected group assessed on both days 1 and 3 (Fig. 15B).

The eNOS protein expression in BOECs was significantly enhanced by Col. I knockdown on both day 1 and day 3 (1.7 ± 0.1 and 1.7 ± 0.3 fold increase on day 1 and 3, respectively, compared to scramble siRNA transfected BOECs on POLY on day 1,) (Fig. 16A). This finding was further confirmed by increased eNOS mRNA level after Col. I siRNA transfection (2.9 ± 0.9 fold increase compared to control) (Fig. 16B). This effect can be attenuated when Col. I siRNA transfected BOECs are cultured on Col. I coating surfaces, especially on day 3 (1.7 ± 0.3 vs. 1.0 ± 0.1 for Col. I siRNA transfected BOECs

cultured on POLY and Col. I, respectively) (Fig. 16A), confirming a strong extracellular matrix mediation on eNOS expression in BOECs.

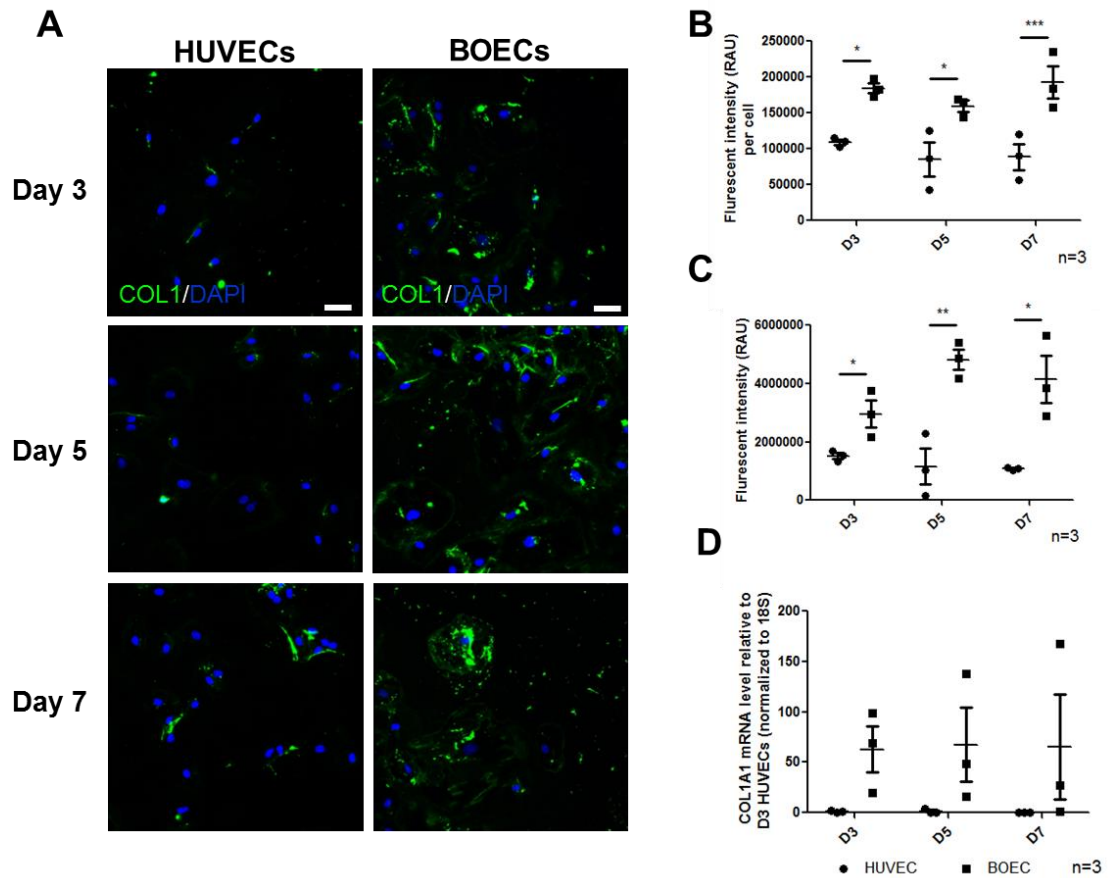


Figure 13: Basal Col. I expression in HUVECs and BOECs. Cells were cultured on polystyrene and on days 3, 5, and 7, cells were fixed and stained by Col. I antibody and DAPI to locate nuclei (A). Scale bar indicates 50 μ m. The total and average fluorescent intensity of Col. I staining were plotted (B and C). The mRNA samples were harvested from different cells on indicated times and Col. I mRNA level were evaluated (C). * and *** indicate $p < 0.05$ and $p < 0.001$, respectively. n indicates the experimental replicates.

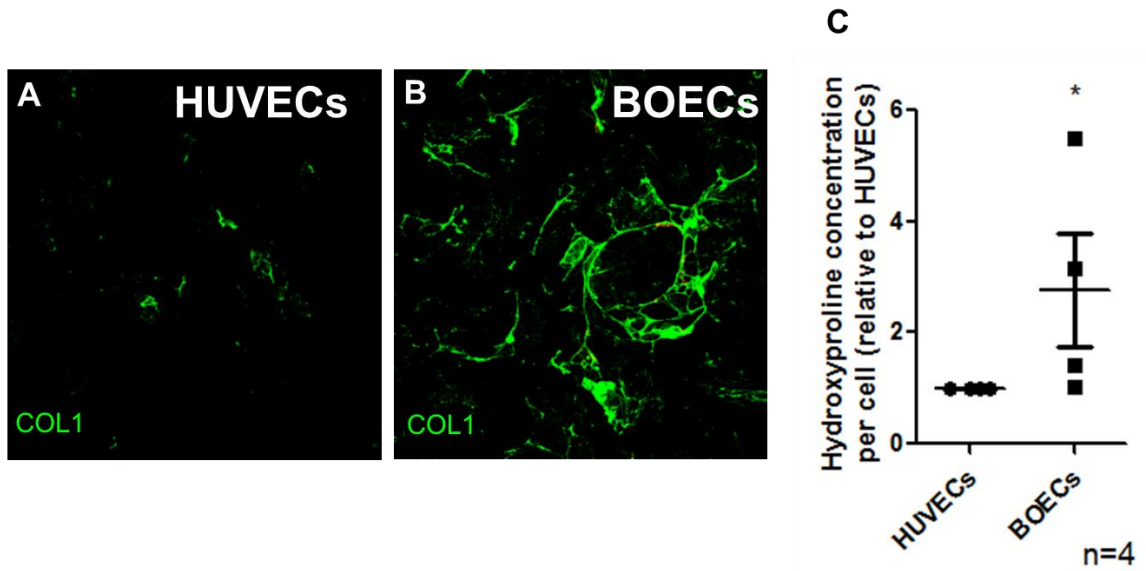


Figure 14: Basal Col. I depositions in HUVECs and BOECs. Cells were cultured in the same condition until 80% confluence followed by decellularization with 0.4% Triton X in 50 mM NH₄OH buffer. Remaining matrix on the plate was stained with Col. I antibody (A and B). The concentration of hydroxyproline in the decellularized matrix was measured (C). n indicates donor number. * indicates p < 0.05.

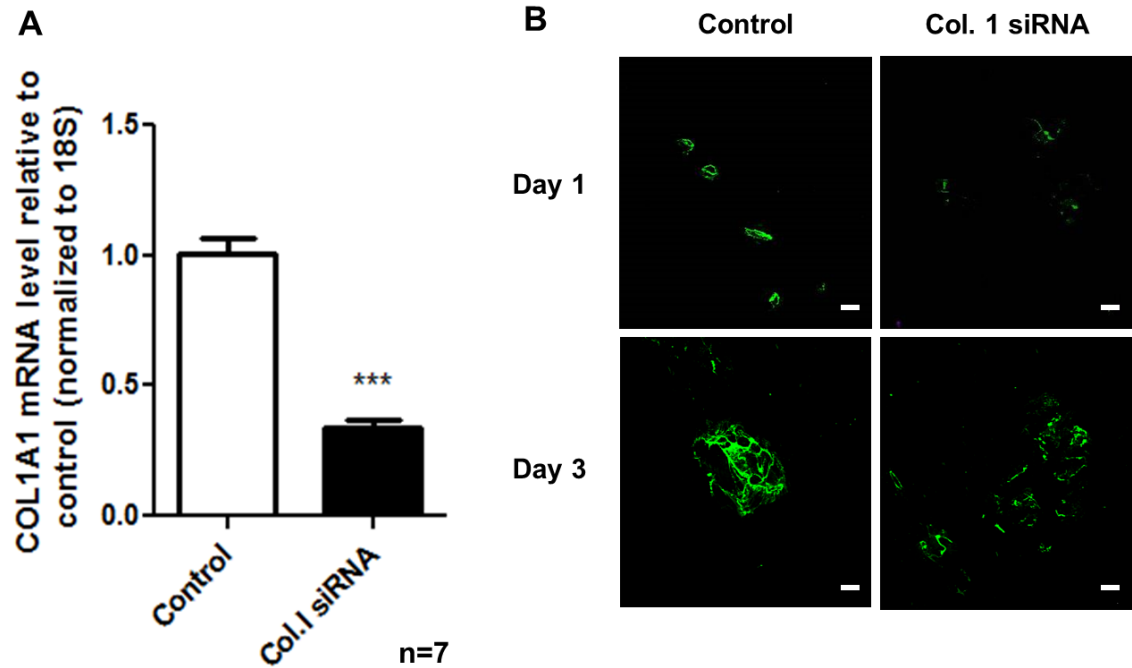


Figure 15: Col. I siRNA transfection blocked Col. I expression in BOECs. Cells were transfected with Col. I siRNA or scramble siRNA for 24 hours followed by qRT-PCR measurement of Col. I mRNA expression (A). To measure the effect on Col. I protein expression, Cells were first undergone transfection process followed by re-plate onto POLY surface. On day 1 and day 3, cells were decellularized and stained with collagen I antibody (B). *** indicates $p < 0.001$. Scale bar = 20 μm .

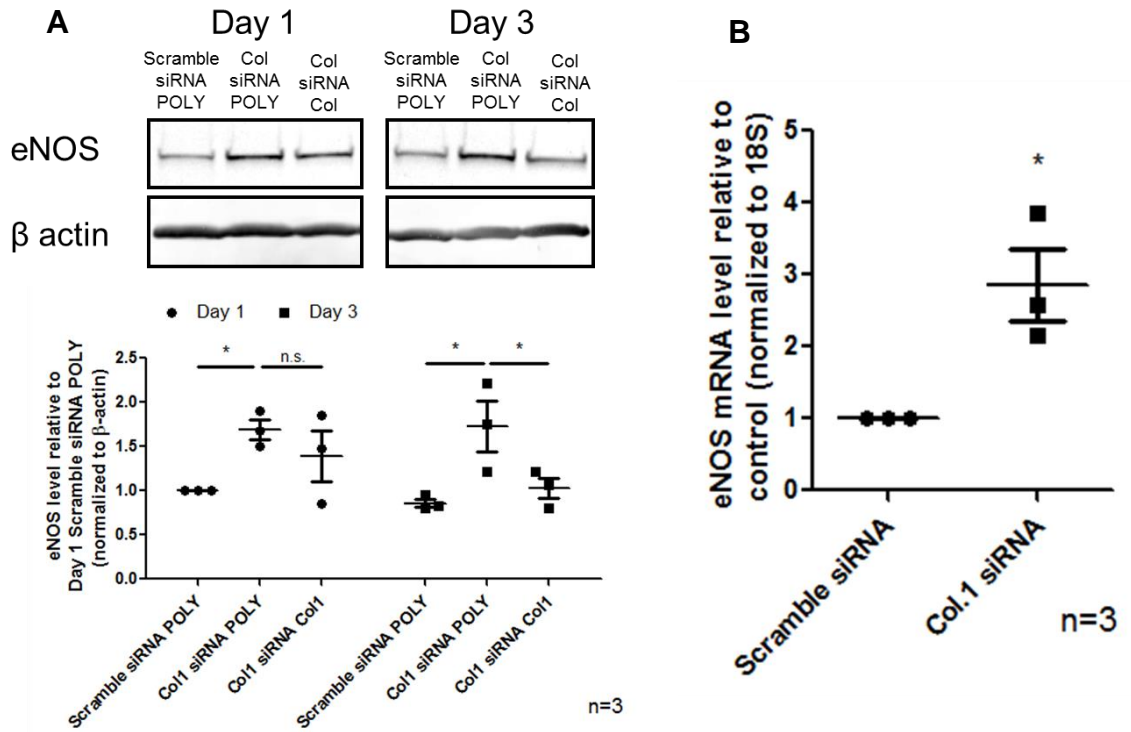


Figure 16: Col. I siRNA and eNOS expression in BOECs. Cells were transfected with Col. I siRNA or scramble siRNA and cultured for 24 hours followed by re-plating on POLY or Col. I coated surfaces, respectively. On days 1 and 3, western blot was performed to evaluate eNOS protein expression of each condition (A). The qRT-PCR was performed to measure eNOS mRNA expression on day 1 after re-plating. * indicates $p < 0.05$.

3.5. Discussion

In this study, a potential mechanism that may elucidate the cause of reduced eNOS level in BOECs has been proposed. I have demonstrated here that eNOS expression and activity levels in BOECs were markedly reduced compared to mature ECs. When cultured on different ECM coated surfaces, the eNOS level of HUVECs, but not BOECs, were significantly downregulated compared to cells on polystyrene. Interestingly, when cultured on polystyrene, BOECs express significantly higher level of Col. I compared to mature ECs. Blocking Col. I synthesis significantly enhanced eNOS expression in BOECs. Taken together, these results suggest BOECs contain lower eNOS level compared to mature endothelial cells partially due to their higher Col. I deposition.

Endothelial NOS catalyzes the production of NO and mediates a variety of angioprotective functions in vivo including endothelial migration, neovascularization, blood vessel dilation, thrombosis and platelet adhesion and as such is an ideal candidate gene for restoring endothelial functional activity. However, my data showed that BOECs express lower eNOS protein and gene expression levels when compared to mature ECs, suggesting the existence of different regulation mechanisms in these cells. Endothelial NOS has been widely demonstrated to be regulated through either transcriptional or post-transcriptional stimulations (reviewed in [238]). I have measured the eNOS mRNA degradation rate and found markedly decreased eNOS half-life in BOECs compared to that in HUVECs, indicating that the reduced eNOS levels may be partially due to decreased eNOS mRNA stability. Many stimuli were proposed to post transcriptionally regulate eNOS signaling including shear stress, hypoxia, thrombin, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, and ECM (reviewed in [238]). However,

in my culture system, the primary stimulus is the extracellular matrix secreted by cells, which I have further investigated.

ECM is a collection of extracellular molecules that provides structural support and regulates cell adhesion and cell signaling. Many studies have demonstrated the tight correlation of cell-ECM interactions and eNOS signaling [180-182]. Compared to normal basal membrane ECM components (e.g. Col. IV), abnormal ECM protein (e.g. Col. I) in vessel walls could be responsible for the decreased eNOS expression and activity levels through integrin-linked kinase (ILK) dependent pathways [180]. FN and LAMA which are the main ECM components, have both been shown to negatively regulate eNOS expression and activity possibly through p38 MAPK dependent pathway in endothelial cells [181,182]. In line with previous findings, after 3 days culture on Col. I, FN, LAMA, and Col. IV, the eNOS protein and mRNA expressions in HUVECs was significantly reduced compared to control, suggesting a strong matrix mediated eNOS regulation in mature ECs. Interestingly, the regulation of eNOS expression was similar across various ECM substrates, suggesting that eNOS regulation may be ECM component-independent or be dependent on a cell adhesion pathway common to all of the tested substrates.

Unlike the eNOS regulation in HUVECs, when cultured on different ECM coated surfaces, the eNOS level of BOECs was unchanged compared to control. I have also studied the effect of ECM on cell proliferation and survival and found that when cultured on specific ECM coated surfaces a significant increase in cell survival and proliferation can be obtained in HUVECs as compared to HUVECs on polystyrene whereas in BOECs, no significant change was observed on different substrates. One of the possible reasons is

that BOECs may highly produce ECM proteins that exert regulation on signaling transduction in the control group, which minimizes the effect of additional ECM substrates. Another possible reason is that the cell-deposited ECM proteins may also form a barrier that prevents the intracellular regulation by external ECM substrates. A previous report has demonstrated BOECs highly deposited ECM proteins (e.g. Col. IV, FN, and LAMA) that assemble to an organized web-like structure whereas mature ECs (e.g. HUVECs and human umbilical artery ECs) only express these ECM proteins intracellularly [237]. Similar to this finding, my study has demonstrated that the Col. I expression is markedly enhanced in BOECs compared to mature ECs upon initial culture and gradually increased during culture. Staining of decellularized deposited matrix has revealed that the highly deposited Col. I in BOECs can form a mesh-like structure whereas in HUVECs, no organized structure was observed. Unlike my data, Kusuma et al., have shown that Col. I expression was not detected in all of their cultures but Col. IV, FN, and LAMA expression was found higher in BOECs [237]. The discrepancy may be due to the cell source where I harvested from peripheral blood and Kusuma et al., isolated from umbilical cord blood. The different cell origin (e.g. adult vs. fetal) may have a great impact on protein regulations. Another possible reason might be the culture conditions where I cultured the cells on POLY whereas Kusuma et al., expanded cells on Col. I coated surfaces. It is widely accepted that coating substrates exert great influence on cell behaviors and differentiation and can modify numerous gene and protein expression. For instance, previous study has demonstrated that the expression levels of FN, LAMA, thrombospondin-1, and Col. IV in tracheal epithelial cells were changed when cultured on Col. I gel-coated membranes compared to control [239]. To further investigate the

regulation of eNOS in BOECs, I have blocked the Col. I expression by Col. I siRNA transfection. With the reduced Col. I mRNA and protein expressions, the level of eNOS in BOECs was significantly enhanced and this effect was attenuated when the Col. I siRNA transfected cells were cultured on Col. I coated surfaces, confirming a matrix mediated effect on eNOS signaling in BOECs.

Taken together, these findings indicate the regulation of eNOS by ECM protein is similar in BOECs and mature ECs and the reduced eNOS expression level in BOECs is due to highly deposition of Col. I. To my knowledge, this is the first study of the regulation of eNOS by ECM substrates in BOECs. Recent work has used RNA sequencing analysis to study pulmonary endothelium in patients with pulmonary arterial hypertension and demonstrated that the expression of Col. IV is downregulated whereas the eNOS expression was upregulated [240], suggesting a reversed regulation between eNOS and Col. IV in vivo. This study gives us an indication of using the novel knowledge presented here to identify biomarkers for cardiovascular disease diagnosis.

**CHAPTER 4: THE ROLE OF INTEGRIN
SIGNALING AND FOCAL ADHESION
COMPLEXES ON ENOS REGULATION IN
BOECS**

4.1. Introduction

In Chapter 3, I have described that the regulation of eNOS expression by ECM substrate is similar in HUVECs and BOECs and the reduced level of eNOS in BOECs may be due to its highly Col. I deposition. I have also observed that the negative regulation of eNOS is similar across various ECM substrates suggesting the existence of an ECM component-independent mechanism. Adherent cells bind to ECM substrates through transmembrane heterodimeric proteins called integrin that is composed of one alpha subunit and one beta subunit. There are 18 α and 8 β subunits and currently 24 combinations in total in mammals (reviewed in [241]). Collagens, the most abundant proteins in connective tissue, primarily bind to integrin $\alpha1\beta1$, $\alpha2\beta1$, $\alpha10\beta1$, and $\alpha11\beta1$ [242]. Several studies have shown the key role of integrins on eNOS regulation [180,182,243]. Yang et al have demonstrated that blockage of $\beta1$ integrin activation inhibited the shear induced signaling involved in Src-family kinase, PI3-kinase, Akt and eNOS on the bovine aortic endothelial cell surfaces [243]. When treated with $\beta1$ integrin antibody, studies by Viji et al and Gonzalez-Santiago et al have shown the effect of FN and Col. I on eNOS expression and activity is attenuated indicating that the regulation of eNOS by ECM substrates may be through an integrin dependent mechanism [175,177].

Integrin binding to ligands results in focal adhesion complex formation which induces the recruitment of focal adhesion (FA) proteins such as focal adhesion kinase (FAK). FAK is part of the FA complex involved in the regulation of cell mechanical homeostasis and other biological processes such as proliferation, differentiation, and migration and recently been shown to regulate eNOS signaling [244-247]. Koshida and colleagues have demonstrated that the flow-induced dilation of rat coronary arterioles was impaired by

inhibition of FAK autophosphorylation site. They have also observed that FAK inhibition reduced flow-induced phosphorylation of Akt at Ser473 and eNOS Ser1179, which is consistent with preventing flow-induced activation of Akt and eNOS [246], suggesting the key role of FAK on eNOS activation. The assembly of a multiprotein complex to the cytoplasmic domain of integrin further binds to actin cytoskeleton and initiates its polymerization that is capable of signal transduction to nuclei (reviewed in [248]). An association between actin cytoskeleton organization and eNOS expression/activity has previously been described [183][184][185][186]. Endothelial cells exhibited decreased actin stress fiber formation after dominant-negative RhoA mutant overexpression resulted in increased eNOS expression and activity. Mice treated with a Rho inhibitor or the actin cytoskeleton disrupter cytochalasin D showed increased vascular eNOS expression and activity, leading to a decrease in cerebral infarction size after middle cerebral artery occlusion [184]. Su et al., have demonstrated that incubation of purified eNOS with either G-actin increase the eNOS activity higher than that with F-actin. Treatment with swinholide A, an actin filament disruptor, increased in eNOS activity, eNOS protein content in PAECs. In contrast, incubation of PAECs with phalloidin, an actin filament stabilizer decreased in eNOS activity and expression [186]. These data suggests a negative regulation of eNOS signaling by stress fiber formation. In this Chapter, I hypothesized that the regulation of eNOS expression by ECM proteins in HUVECs and BOECs is through a β 1 integrin, FAK and actin cytoskeleton polymerization dependent mechanism.

4.2. Specific Aims

- To measure the differential expression of integrins ($\alpha 1$, $\alpha 2$, $\alpha 5$, $\beta 1$, $\beta 3$) and correlate these to the number of focal adhesion sites, cell spreading and area in BOECs, HUVECs, and HAECs.
- To demonstrate eNOS regulation by Col. I substrate via a $\beta 1$ integrin/FAK/actin polymerization dependent mechanism in BOECs and HUVECs.
- To determine the role of Rho associated kinases on the eNOS bioactivity in BOECs.

4.3. Methods and Materials

4.3.1. Western blot

For protein (eNOS, integrin, and nitrotyrosine) detection, Nupage 3-8% Tris-Acetate gels were used as previously described [2]. To extract whole cell protein, HUVECs, HAECs, and BOECs were lysed and collected by cell scraper in cold RIPA buffer supplemented with protease inhibitor (Roche Applied Sciences, Indianapolis, IN), sodium fluoride and sodium orthovanadate (Sigma Aldrich, Oakville, ON, Canada). Protein concentrations were determined using lowry assay (Bio-Rad) and 30 – 40 μg of total protein mixed with LDS sample buffer, heated to 70 °C (10min), and the reducing agent (DTT) was then added. After gel transfer, nitrocellulose membrane were blocked with 5% skim milk and incubated with following primary antibodies: integrin $\beta 1$, $\beta 3$, αv , $\alpha 5$, $\alpha 1$, and $\alpha 2$, eNOS, nitrotyrosine or β -actin as control at 4 °C overnight. The IRDye 800CW and IRDye 680RD secondary antibodies were used for visualization on an Odyssey Imaging System.

4.3.2. Immunocytochemistry

To detect cytoskeleton and focal contact contacts, 1×10^4 cells/well were inoculated into 12 well-plate containing 18 mm cover slips (either coated or non-coated). After 3 days of culture, adherent cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton-X in PBS for 3 min, and blocked with 5% FBS PBS for 1 hour at RT. Focal contact sites were probed with a monoclonal anti-vincullin antibody (Sigma Aldrich, Oakville, ON, Canada) overnight at 4 °C, and 1 hour incubation with a secondary antibody conjugated with Alexa Fluor 488 followed by staining with rhodamine phalloidin (20 min, Life Technologies, Burlington, Ontario, Canada) and DAPI (5 min). All samples were washed (PBS), mounted, and assessed with confocal microscopy (Olympus Fluoview FV1000). To quantify the focal adhesion sites, cylindrical points (vinculin stain) on the edge of actin filaments in the individual cells were counted. The average of 50 represented cells was used to plot cell projected area and focal adhesion sites graphs in each condition.

4.3.3. Quantitative Real-time reverse transcription-PCR

Total mRNA was extracted from different cells in indicated conditions by using RNA isolation system (Exiqon, Woburn, MA). The cDNAs were synthesized using a cDNA synthesis kit (Life Technologies, Burlington, ON, Canada). The mRNA levels of eNOS and Col. 1 was analyzed using Taqman primer (Life Technologies) and Real-Time PCR system (Bio-Rad). Relative changes in mRNA expression of target genes were determined using $\Delta\Delta C_t$ method normalized to 18S.

4.3.4. Silencing interference RNA (siRNA) Transfection

1×10^5 BOECs or HUVECs/well was inoculated into 6 well plates, cultured for 24 h followed by transfection with indicated siRNA ($\beta 1$ integrin siRNA or FAK siRNA) or scramble control (200 μ L transfection mix into each well) as per manufacturer's instructions (100 pmol siRNA, 200 μ L buffer, and 4 μ L of jetPRIMETM, Polyplus Transfection, New York, NY). The media was replaced 4 h after transfection and the transfected cells were re-plated 24 hrs after. For experiment on HUVECs, transfected cells were re-plated and cultured on various ECM substrates for additional 3 days before analysis of eNOS level. For experiment on BOECs, transfected cells were re-plated and cultured on POLY for 1 day prior to further assessments.

4.3.5. ECM protein coating and cell culture

The procedure of ECM coating and cell culture is the same as the one described in Chapter 3. In brief, same concentrations (15 μ g/mL) of fibronectin (FN, R&D Systems, Minneapolis, MN), collagen I (Col. I, BD Biosciences, San Diego, CA) were prepared in PBS and surfaces were incubated at room temperature (RT) for 2 hours.

4.3.6. The effect of actin polymerization on eNOS signaling

To assess the effect of actin polymerization on eNOS signaling in HUVECs, cells were treated with ROCK inhibitors (fasudil (10 μ M) or Y27632 (25 μ M)) for three days while cultured on FN or Col. I coated surfaces (POLY was used as control) followed by eNOS assessment. To test the regulation of actin polymerization on eNOS signaling in BOECs, cells were treated either with ROCK inhibitor (Y27632 (25 μ M)) or cytochalasin D (1 μ M) for 24 hrs while cultured on POLY prior to further assessments.

4.3.7. Superoxide detection

The intracellular content of O_2^- was quantified using dihydroethidium (DHE, Life Technologies). Cells were exposed to Y27632 (25 μ M) or O_2^- scavenger polyethylene glycol-superoxide dismutase (PEG-SOD; 40 U/L; Sigma) for 24 hours prior to exposure to DHE and maintained in the presence of the scavenger of the whole course of the experiment. Cells were exposed to DHE (25 μ M) for 20 minutes, and the regular medium replaced for an additional hour prior to processing for flow cytometry or fluorescent microscopy. FL2 channel was used for DHE during flow cytometry. The superoxide donor (Menadione, Sigma) was used as negative control.

4.3.8. Statistical Analysis

Comparison between groups was performed with Student's t test or for multi-group comparisons by ANOVA followed by Tukey's post hoc test. All analysis was performed with GraphPad Prism 5.0; values shown graphically are mean \pm standard error.

4.4. Results

4.4.1. Integrin expression, focal adhesions, and actin polymerization in BOECs and HUVECs

To evaluate the integrin expression, BOECs and HUVECs were cultured under the same condition for 3 days and cell lysates were harvested and assessed by western blot. There were 3 batches of HUVECs and 4 donors of BOECs used in this experiment. I found multiple integrin expression such as β 1, β 3 and α v integrin (1.80 ± 0.19 , 1.89 ± 0.46 , and 2.20 ± 0.46 fold increases for β 1, β 3 and α v integrin, respectively) in BOECs is

significantly higher compared to that in HUVECs (Fig. 17). To assess the focal adhesions and actin cytoskeleton, HUVECs, HAECs and BOECs were stained with phalloidin (cytoskeleton) and anti-vinculin antibody (focal adhesion sites) (Fig. 18A). I have found that BOECs display significantly more focal adhesion sites (57.41 ± 6.68 vs. 15.36 ± 1.55 vs. 19.63 ± 1.63 focal adhesion sites per cell for BOECs, HUVECs and HAECs, respectively) (Fig. 18B) and larger projected area (3.43 ± 0.64 fold increase for BOECs relative to HUVECs) (Fig. 18C) compared to HUVECs and HAECs. There is no significant difference in focal adhesion sites per area among these cells (Fig. 18C). The focal adhesion sites (45.95 ± 0.32 vs. 33.56 ± 2.99 per cell for control and Col. I siRNA transfected cells, respectively) and cell projected area (2774 ± 238.7 vs. $2071 \pm 65.92 \mu\text{m}^2$ for control and Col. I siRNA transfected cells, respectively) was significantly reduced when cells were transfected with Col. I siRNA (Fig. 19). Furthermore, much less stress fiber has been observed in Col. I siRNA transfected cells, suggesting a key regulation of Col. I deposition on both focal adhesion and actin cytoskeleton.

4.4.2. The effect of $\beta 1$ integrin on eNOS signaling

To assess the regulation of $\beta 1$ integrin on eNOS signaling, HUVECs were transfected with $\beta 1$ integrin siRNA and cultured on FN or Col. I coated surfaces. Significant increase of eNOS expression (1.51 ± 0.18 fold increase compared to scramble siRNA) was found when $\beta 1$ integrin was blocked on Col. I coated surface whereas modest difference was found when $\beta 1$ integrin-transfected cells were cultured on FN coated surface (Fig. 20A). For BOECs, cells were transfected with $\beta 1$ integrin siRNA and re-plated on POLY coated surface. There was a modest but significant increase of eNOS expression (1.26 ± 0.09

fold increase) when cells were transfected with $\beta 1$ integrin siRNA compared to scramble control.

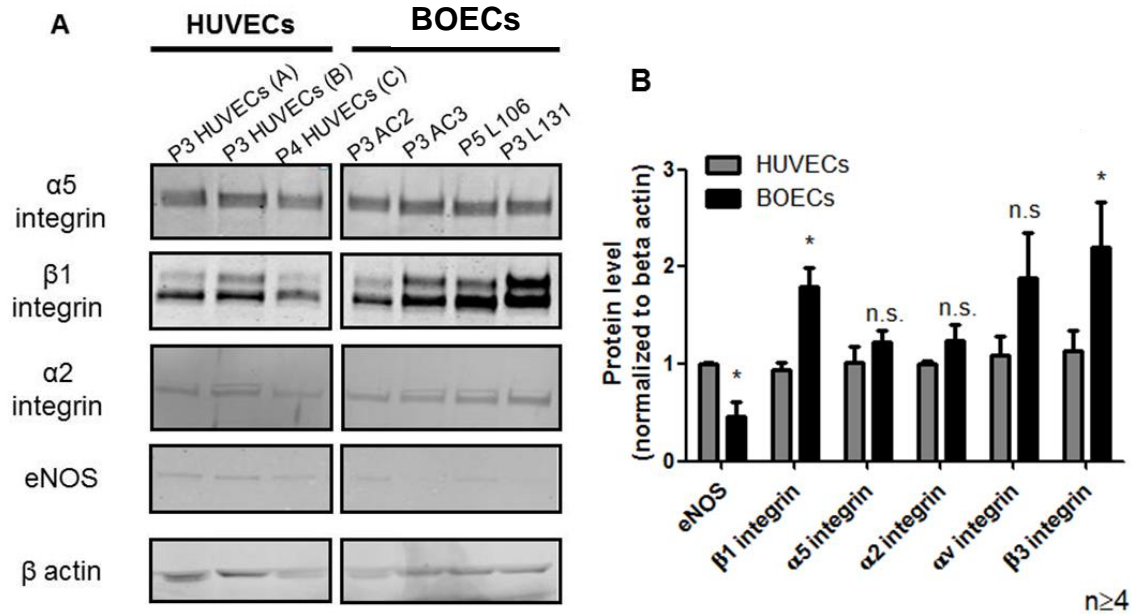


Figure 17: Comparison of integrin and eNOS expressions in HUVECs and BOECs. Three batches of HUVECs and four donors (AC2, AC3, L106, and L131) of BOECs were used. Cells were cultured for 3 days and cell lysates were harvested for protein assessment on integrin subunits ($\beta 1$, $\beta 3$, $\alpha 2$, $\alpha 5$ and αv). * indicates $p < 0.05$.

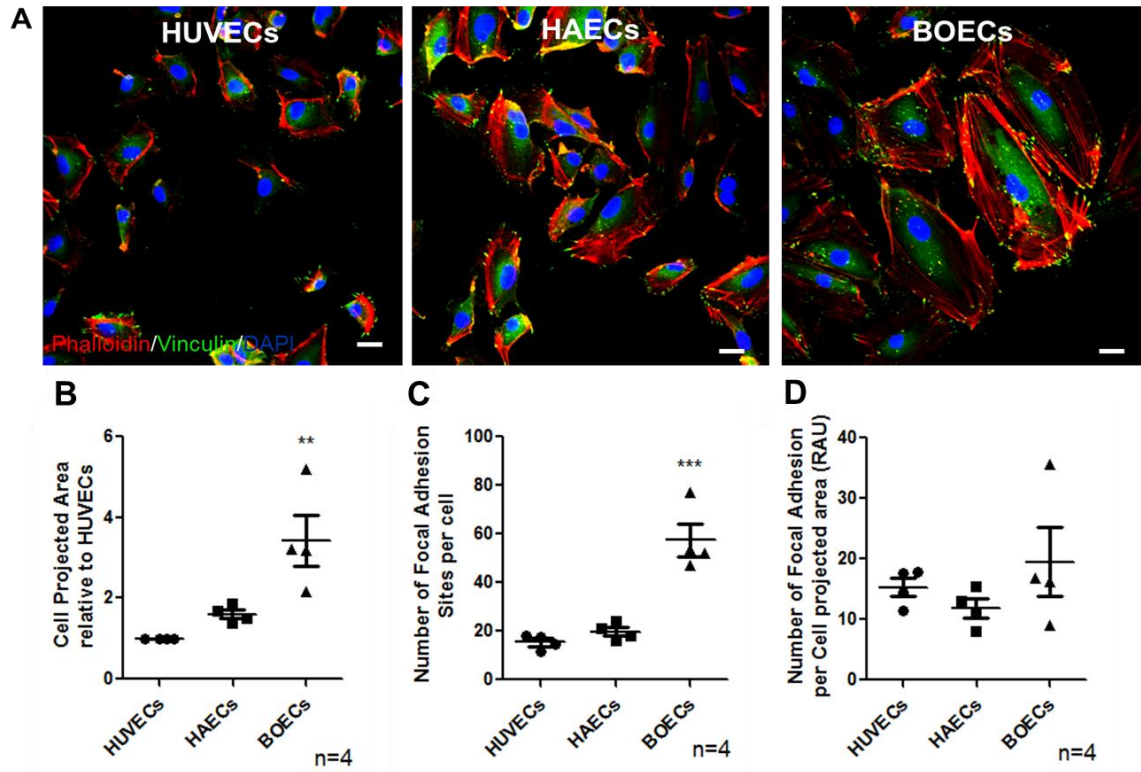


Figure 18: The focal adhesion and actin polymerization in HUVECs, HAECs, and BOECs. Cells were cultured for 4 days followed by further assessments. Cytoskeleton and focal adhesion were stained by phalloidin (rhodamine) and vinculin antibody (secondary antibody conjugated with Alexa fluor 488), respectively. DAPI was used as nuclei staining (A). The size of cell area (B) and number of focal adhesion sites (C) were measured. The number of focal adhesion per cell area was calculated (D). Scale bar indicates 10 μm . ** and *** indicate $p < 0.01$ and $P < 0.001$, respectively.

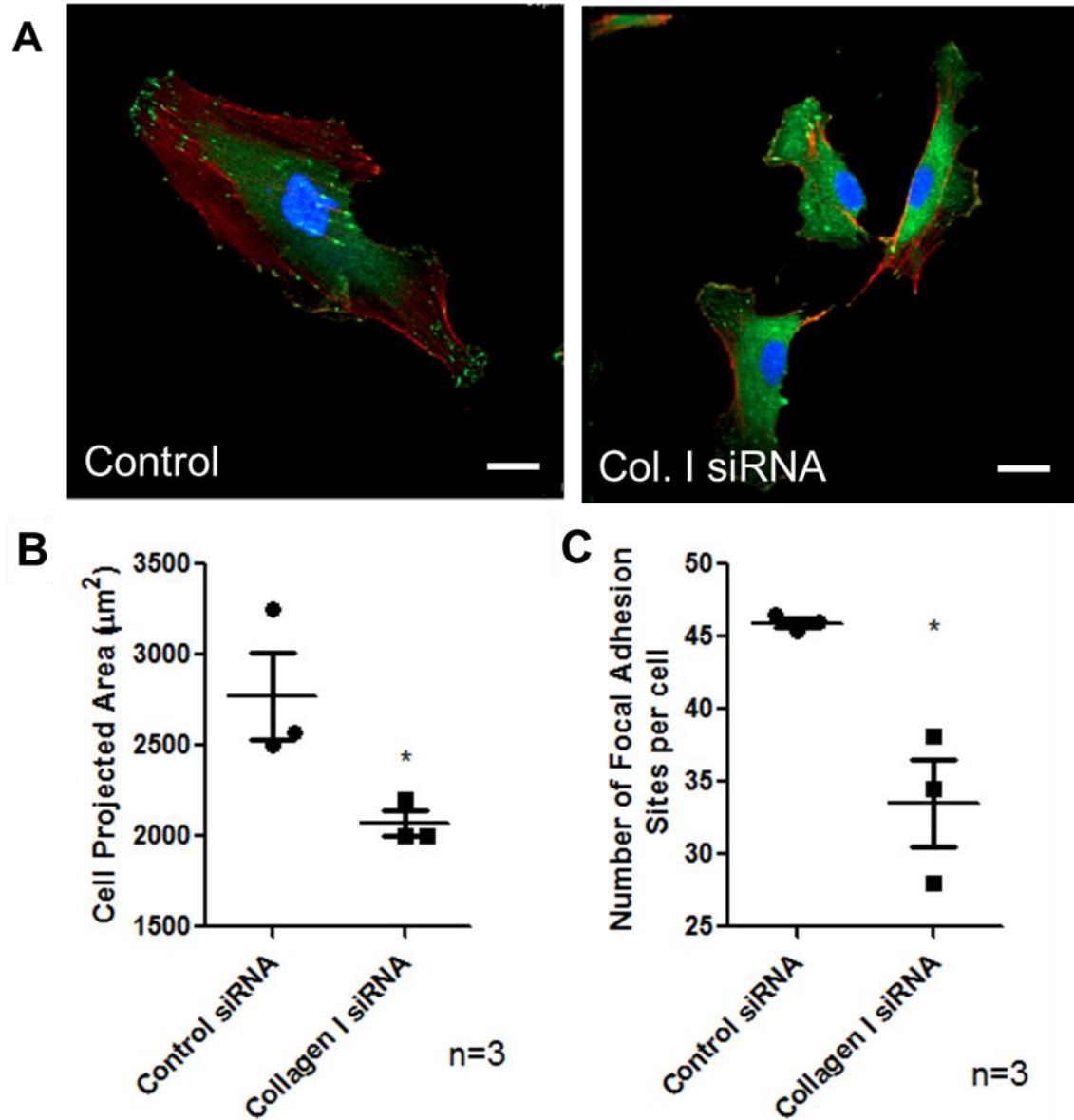


Figure 19: The focal adhesion and actin polymerization in BOECs after Col. I siRNA transfection. Cells were transfected with Col. I siRNA or control siRNA. After 24 hours, cells were re-plated onto POLY for 24 hours. Cytoskeleton and focal adhesion were stained and imaged by fluorescent microscopy (A). The size of cell projected area (B) and number of focal adhesion sites (C) in different conditions were measured. Scale bar indicates 10 µm. * indicates $p < 0.05$.

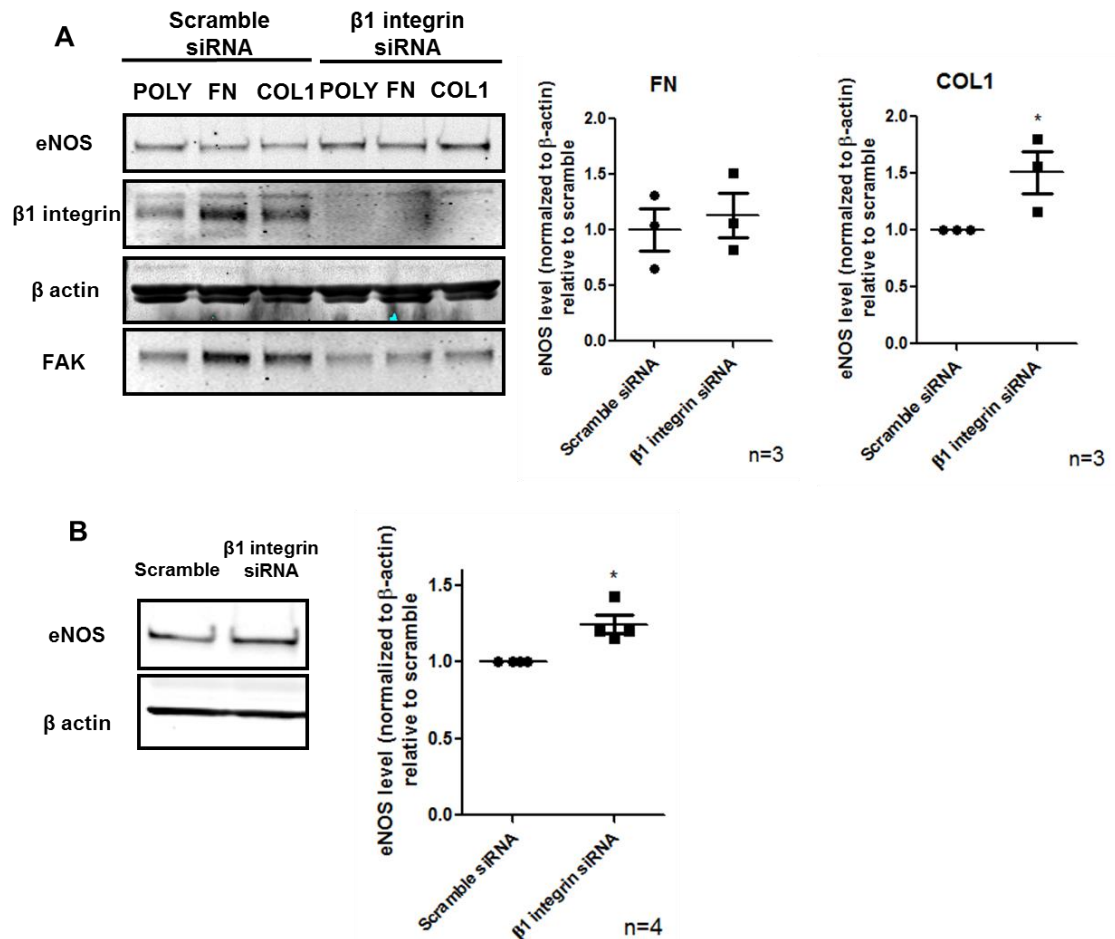


Figure 20: The effect of β1 integrin on eNOS regulation in HUVECs and BOECs. HUVECs were transfected with scramble vector or β1 integrin siRNA followed by culturing on POLY, FN, or Col. 1 coated surfaces for 3 days. Western blot was applied to detect eNOS, β1 integrin, FAK expression at the end of experiment (A). For BOECs, cells were transfected with scramble siRNA and β1 integrin siRNA for 24 hours and replated on POLY for additional 24 hours followed by western blot assessment on eNOS protein expression (B). * indicates $p < 0.05$.

4.4.3. The effect of focal adhesion kinase on eNOS signaling

I have noticed that focal adhesion kinase level in HUVECs was enhanced on FN or Col. I compared to that on POLY. This effect was attenuated when β 1 integrin was blocked, suggesting that FAK is the downstream target of β 1 integrin (Fig. 20A). After FAK siRNA transfection, significant increase of eNOS expression (1.99 ± 0.36 fold increase) was obtained in HUVECs on Col. I coated surface compared to scramble siRNA control (Fig. 21A). Similar effect was found in BOECs. After 24 hours of re-plating, there was a significant increase of eNOS expression (1.71 ± 0.14 fold increase) when cells were transfected with FAK siRNA compared to scramble control (Fig. 21B).

4.4.4. The effect of actin polymerization on eNOS signaling

After FAK siRNA transfection, I have found a marked reduction of cell projected area as well as focal adhesion sites in BOECs. Additionally, the amount and length of actin filaments are markedly reduced after FAK blockage (Fig. 22), suggesting a significant role of FAK on actin polymerization.

I have then studied the effect of actin polymerization on eNOS signaling. When cultured on Col. I and FN, the expression level of eNOS in HUVECs can be significantly enhanced by ROCK inhibitor (Y27632) treatment (1.83 ± 0.31 and 1.64 ± 0.28 fold increases compared to control (without Y27632) for Col. I and FN, respectively) (Fig. 23). Similarly, the expression level of eNOS was significantly increased in BOECs when treated with Y27632 (2.35 ± 0.08 (eNOS mRNA) and 1.40 ± 0.11 (eNOS protein) fold increases compared to control) (Figs. 24A and B). ROCK has two isoforms and each exerts different functions. The eNOS expression level was significantly enhanced ($1.37 \pm$

0.07 fold increase compared to control) when ROCK2 was knocked down, however, no difference of eNOS expression was observed when ROCK1 was knocked down (Fig. 24C). Cytochalasin D (Cyto D) is one of fungal metabolites that block polymerization and the elongation of actin. After treatment with Cyto D, the eNOS expression in BOECs is enhanced in a dose-dependent manner (1.94 ± 0.24 (eNOS mRNA) and 1.59 ± 0.13 (eNOS protein) fold increases compared to control when treated with Cyto D ($1 \mu\text{M}$) (Fig. 24D and E).

4.4.5. Nitrotyrosine level in HUVECs and BOECs

In various cardiovascular diseases, such as atherosclerosis and diabetes, eNOS remains as monomer and is not able to bind to BH_4 and produce NO, instead it will generate superoxide. This alteration is called “eNOS uncoupling”. The increased superoxide level reacts with NO and gives rise to peroxynitrite. This substrate can further induce the oxidization of BH_4 to BH_2 and decrease NO production. In this part, I have evaluated the expression level of tyrosine nitration, an indicator of peroxynitrite, in BOECs and HUVECs. Upon culture under the same conditions, the basal level of nitrotyrosine across 3 donors of BOECs is markedly higher (1.92 ± 0.12 vs. 3.20 ± 0.35 for nitrotyrosine expression in HUVECs and BOECs, respectively) while eNOS expression level is markedly lower (1.10 ± 0.15 vs. 0.29 ± 0.19 for eNOS expression in HUVECs and BOECs, respectively) compared to that in HUVECs (Fig. 25).

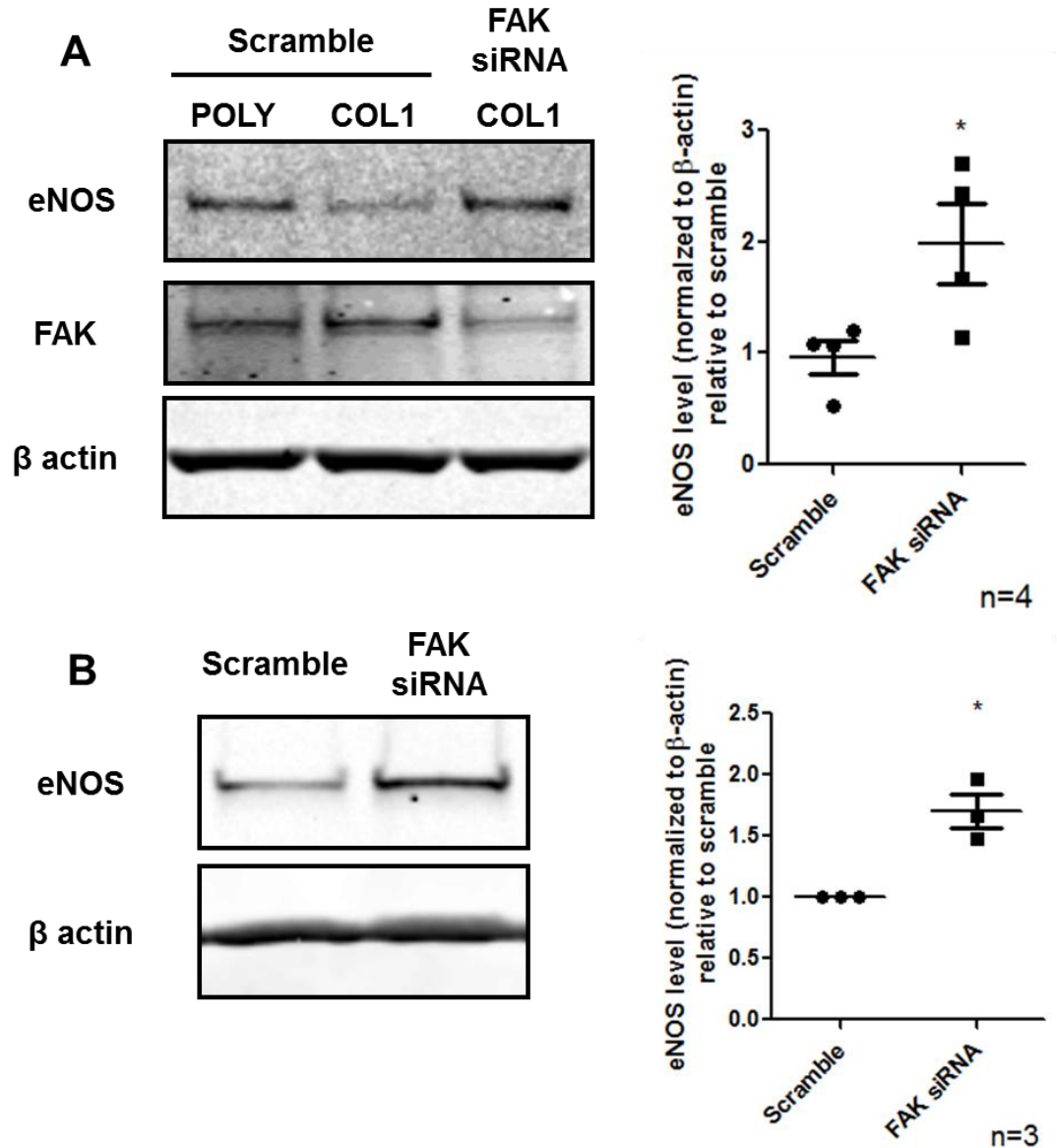


Figure 21: The effect of FAK on eNOS regulation in HUVECs and BOECs. HUVECs were transfected with scramble vector or FAK integrin siRNA followed by culturing on POLY or Col. I coated surfaces for 3 days. Western blot was applied to detect eNOS and FAK expression at the end of experiment (A). For BOECs, cells were transfected with scramble siRNA and FAK siRNA for 24 hours and re-plated on POLY for additional 24 hours followed by western blot assessment on eNOS protein expression (B). * indicates $p < 0.05$. n represents the technical replicates.

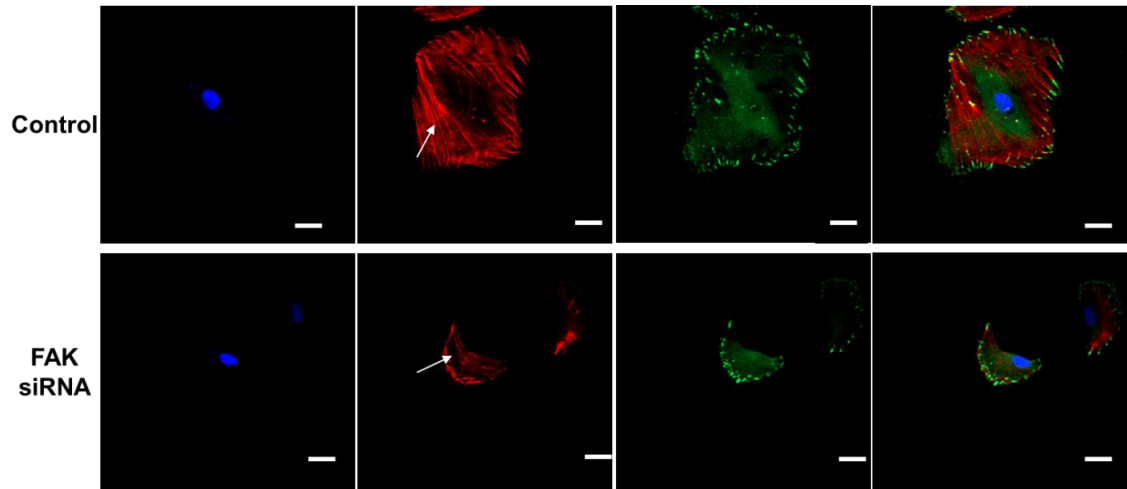


Figure 22: The effect of FAK on focal adhesion sites and actin polymerization. BOECs were transfected with either scramble siRNA or FAK siRNA for 24 hours. Transfected cells were re-plated onto POLY for additional 24 hours and stained for cytoskeleton with phalloidin and focal adhesion sites with anti-vinculin antibody. White arrows indicate actin filaments. Scale bar: 20 μm .

4.4.6. The effect of ROCK inhibitor on eNOS uncoupling

I have then studied the effect of ROCK inhibitor Y27632 on eNOS uncoupling. BOECs were cultured for 24 hours while treated with Y27632 or SOD followed by superoxide and nitrotyrosine detection. The fluorescent microscopy detection revealed that most of the cells were positive for superoxide production in the control group whereas in either Y27632 or SOD treated group, markedly lower amount of cells displayed red fluorescent (Fig. 26A). This finding has been further confirmed by flow cytometry data that shows a markedly decreased superoxide positive staining in groups either treated with Y27632 or SOD compared to control (Fig. 26B). In consistent with superoxide detection, the nitrotyrosine levels in either Y27632 or SOD treated group are markedly reduced compared to control (0.60 ± 0.10 and 0.34 ± 0.04 fold decreases for Y27632 and SOD compared to control, respectively)(Figs. 27A, B, and C). To confirm the correlation between nitrotyrosine and eNOS uncoupling, cells were treated with eNOS uncoupling inhibitor (BH_4) followed by nitrotyrosine detection and I have demonstrated that treatment with $1 \mu\text{M}$ BH_4 markedly decreased tyrosine nitration (Figs. 27D and E).

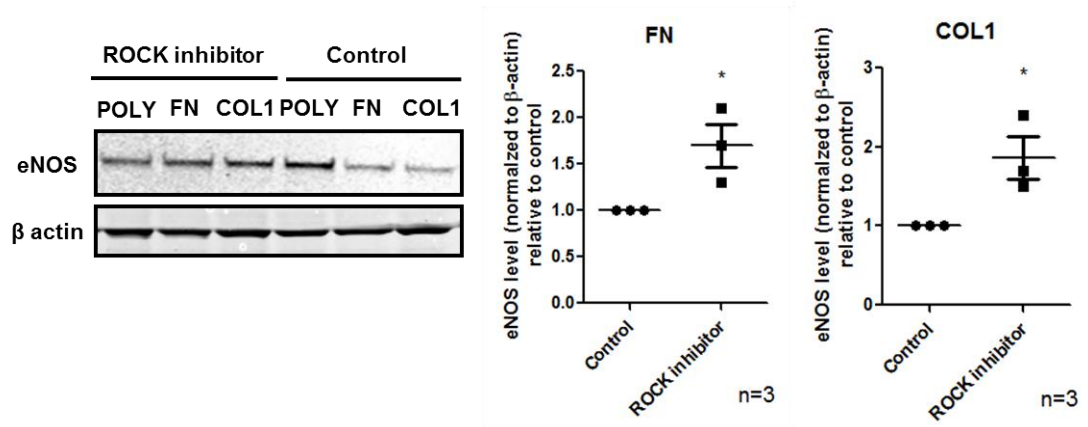


Figure 23: the effect of ROCK inhibitor on eNOS expression in HUVECs in response to ECM substrates. HUVECs were cultured on POLY, FN, or Col. I coated surfaces while treated with 25 μ M ROCK inhibitor (Y27632) for 3 days followed by eNOS protein expression assessment. * indicates $p < 0.05$.

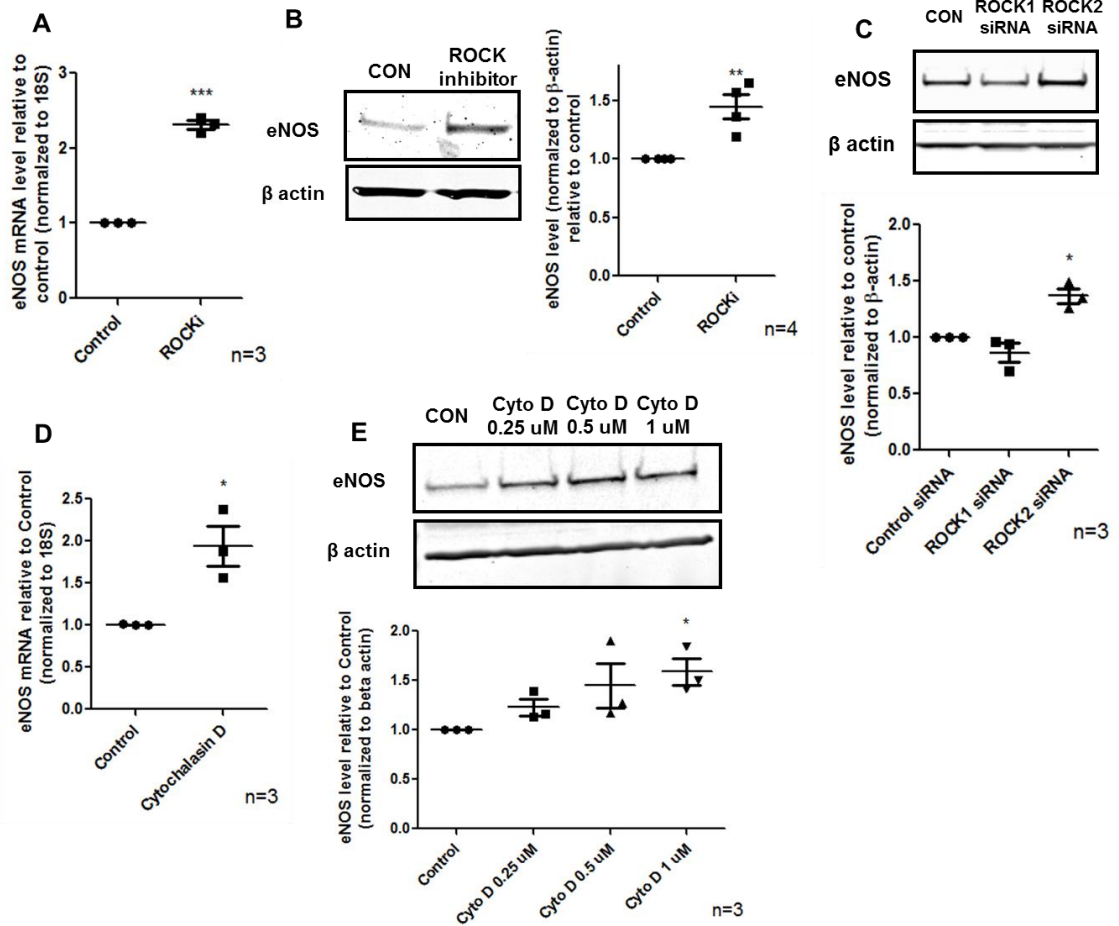


Figure 24: the effect of actin polymerization on eNOS expression in BOECs. Cells were cultured on POLY for 24 hours while treated with 25 μ M Y27632 followed by eNOS gene and protein expression measurement (A and B). To evaluate to effect of ROCK1 and ROCK2, cells were transfected with ROCK1, ROCK2, and scramble siRNA (Control) for 24 hours followed by re-plating. After additional 24 hours culture, protein samples were isolated and assessed by western blot (C). BOECs were first seeded on POLY overnight followed by treatment with cytochalasin D (0, 0.25 μ M, 0.5 μ M and 1 μ M) for 1 hour. After 24 hours of further culture, cell lysates were harvested to measure eNOS gene and protein expression (D and E). *, **, and *** indicates $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

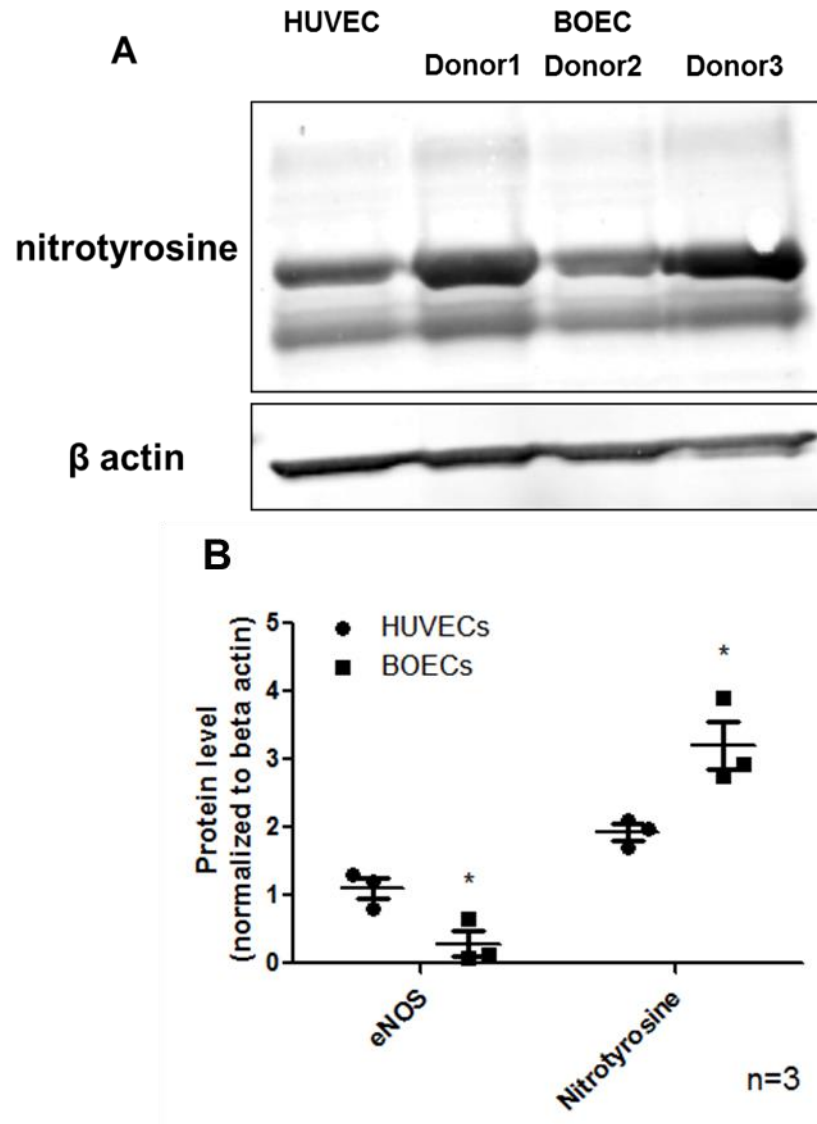


Figure 25: The level of nitrotyrosine in HUVECs and BOECs. Cells were cultured for 3 days followed by harvesting protein lysate. The peroxynitrite level in cell lysates was measured by western blot and nitrotyrosine staining (A). There were 3 donors of BOECs and 1 batch of HUVECs used in the study. The protein expression level of eNOS and nitrotyrosine in HUVECs and BOECs were plotted. n indicates technical replicates. * indicates $p < 0.05$.

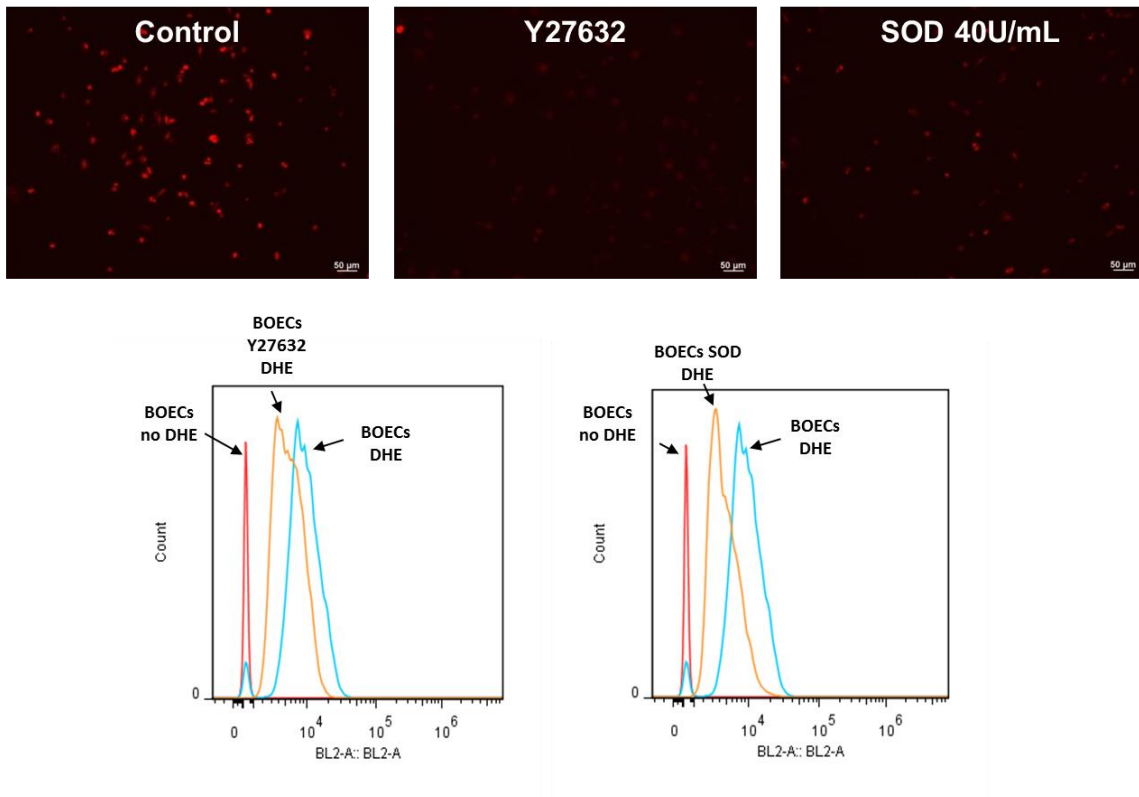


Figure 26: the effect of ROCK inhibitor on superoxide production in BOECs. Cells treated with ROCK inhibitor Y27632 for 24 hours followed by superoxide measurement (Dihydroethidium (DHE) staining, red fluorescent). Fluorescent microscopy (A) and flow cytometry (B) were used to detect superoxide staining in different conditions. Superoxide dismutase (SOD) treated group was used as positive control. Scale bar indicates 50 μm .

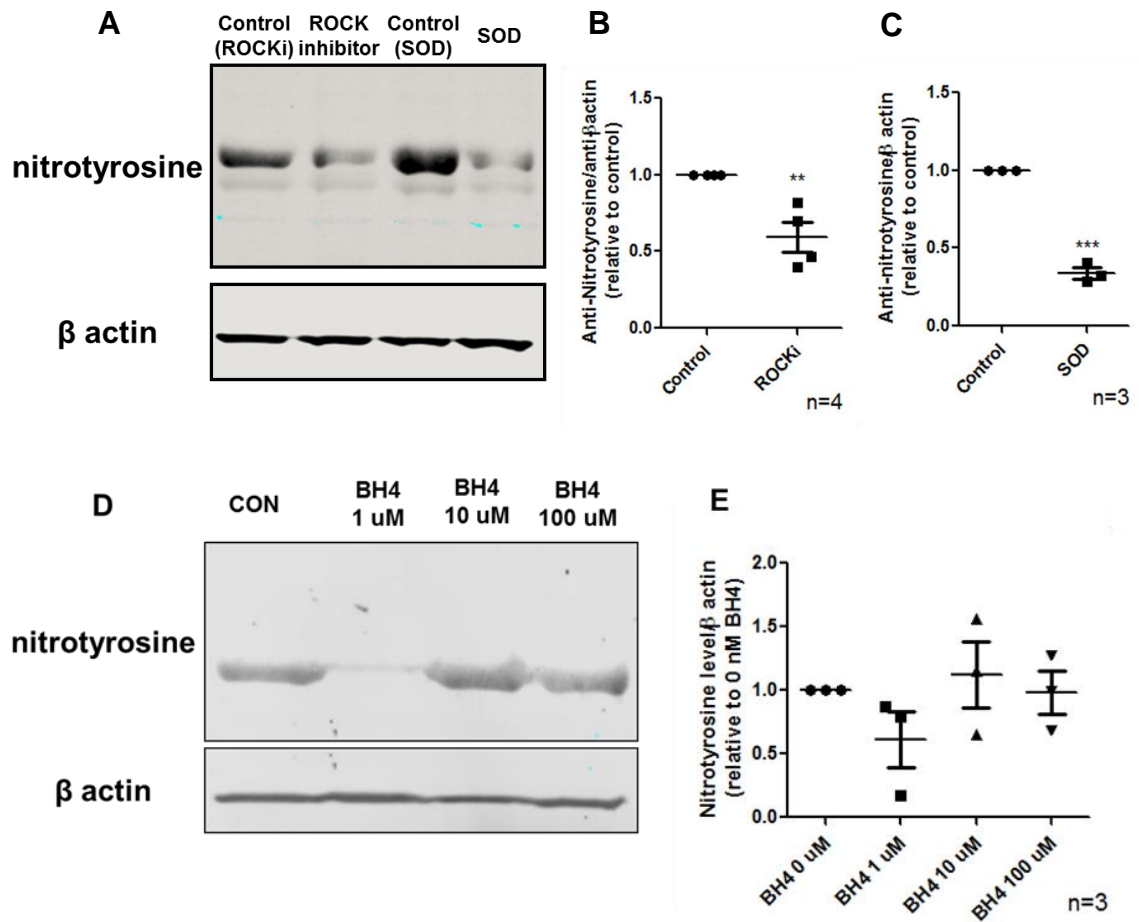


Figure 27: the effect of ROCK inhibitor and tetrahydrobiopterin on peroxynitrite in BOECs. Cells treated with either ROCK inhibitor Y27632 for 24 hours followed by protein isolation. The level of peroxynitrite was detected by western blot and further nitrotyrosine staining (A). SOD (40 U/mL) treated group was used as positive control. The relative nitrotyrosine level in ROCK inhibitor (B) and SOD (C) treated groups were plotted. Cells treated with different concentrations of tetrahydrobiopterin for 24 hours followed by nitrotyrosine detection by western blot (D, E). * and *** indicate $p < 0.05$ and $P < 0.001$, respectively. n indicates technical replicates.

4.5. Discussion

In this study, I have introduced a mechanism on how ECM proteins regulate eNOS expression in BOECs and mature ECs. Consistent with the findings shown in Chapter 3, when cultured under the same condition, BOECs expressed higher levels of integrin and display higher amounts of focal adhesion sites as well as larger cell projected areas compared to that in HUVECs and HAECs, these results are likely due to higher Col. I deposition. The matrix mediated eNOS downregulation can be completely attenuated when either $\beta 1$ integrin or FAK is blocked. Additionally, the inhibition of actin polymerization (Y27632, ROCK2 siRNA transfection or Cyto D) significantly enhances eNOS level in BOECs. These results suggest a negative regulation of eNOS expression level by ECM proteins in endothelial cells via a $\beta 1$ integrin/FAK/actin polymerization dependent mechanism. Further investigation revealed that Y27632 not only enhance eNOS expression level but bioactivity in BOECs.

During most cell-ECM interactions, integrins are induced to adopt the ‘primed’ conformation by recruitment of talin to plasma membrane. The integrin extracellular domains then extend and allow binding specific ECM molecules. Other focal adhesion proteins are subsequently recruited to the integrin-talin platform and form nascent focal complex. The maturation of focal adhesions involves clustering of active, ligand-bound integrins and the assembly of a multiprotein complex that is capable of linking integrins to the actin cytoskeleton and communicating with signaling pathways (reviewed in [248]). In Chapter 3, I have demonstrated that BOECs express higher level of Col. I compared to mature ECs. In this Chapter, I have evaluated the integrin expression, focal adhesion sites and actin cytoskeleton in BOECs and mature ECs. Consistent with the

findings in Chapter 3, markedly higher expression levels of integrins ($\beta 1$ and $\beta 3$), number of focal adhesion sites as well as more and longer actin filament have been observed in BOECs compared to that in mature ECs. Furthermore, the number of focal adhesion sites and actin filament were reduced when Col. I expression/deposition was blocked in BOECs, suggesting that enhanced focal adhesion complex in BOECs is partially due to Col. I deposition.

Integrins act as transmembrane receptors to specific peptide domains of ECM proteins. Three members of the integrin family named $\alpha 1\beta 1$, $\alpha 2\beta 1$, and $\alpha 11\beta 1$ act as receptors for type I collagen and are present in endothelial cells [249]. A previous report has demonstrated that the negative regulation of eNOS expression by a Col. I substrate in HUVECs is attenuated when treated with specific anti- $\alpha 1$, and $\beta 1$ integrin antibodies [180]. Similarly, I have shown here that the reduced eNOS expression in both HUVECs and BOECs due to cell binding to Col. I substrate was reversed by $\beta 1$ integrin blockage. Interestingly, the magnitude of change in eNOS expression after $\beta 1$ integrin inhibition is significant though modest in BOECs. Discoidin domain receptors (DDR) 1 and 2 have been found to serve as receptors for Col. I. and modulate signaling transduction such as metalloprotease expression in response to collagen stimulation (reviewed in [250]). BOECs produced Col. I is dramatically higher than Col. I coating, which may initiate eNOS regulation through a DDR dependent mechanism. I have also noticed that the eNOS expression level has been modestly changed when $\beta 1$ integrin siRNA-transfected HUVECs were cultured on FN. Unlike Col. I, the specific integrin receptors for FN in endothelial cells are predominantly $\alpha 5\beta 1$, and $\alpha v\beta 3$ [251,252], suggesting that FN may

regulate eNOS signaling through $\beta 1$ independent but possibly $\alpha v\beta 3$ dependent mechanism.

FAK is part of the focal adhesion complex involved in the regulation of cell mechanical homeostasis and other processes such as proliferation, differentiation, and migration and recently been shown to regulate eNOS signaling [244-247]. A study by Koshida et al has demonstrated that the flow-induced Akt activation at Ser473 and eNOS activation at Ser1179 of rat coronary arterioles was impaired by inhibition of FAK autophosphorylation site [246], suggesting the key role of FAK in eNOS signaling. In this study, I have first observed that expression of FAK was increased when cells were cultured on FN and Col. I and this effect has been attenuated by $\beta 1$ integrin siRNA transfection, confirming that FAK is the downstream target of $\beta 1$ integrin. Then I have looked at the effect of FAK on eNOS expression. The level of eNOS was significantly enhanced for HUVECs when cultured on both FN and Col. I, confirming the key role of FAK in eNOS regulation by cell-ECM interactions. Consistently, the eNOS expression level was significantly increased in BOECs after FAK was blocked, demonstrating the similar eNOS regulation by Col. I substrate in HUVECs and BOECs.

Actin cytoskeleton plays a critical role in integrin inside out and outside in signaling and regulates signaling communication between focal adhesion complex and nuclei. Its synthesis and polymerization can be regulated by focal adhesion proteins such as FAK. Serrels and colleagues have demonstrated that FAK^{-/-} mouse embryonic fibroblasts were less well spread and displayed few lamellipodia and reduced number of cytoplasmic stress fibres when compared to FAK^{-/-} cells re-expressing wild-type FAK [253]. In line

with previous finding, I have shown here that a significant reduction of stress fiber amount and length as well as the number of focal adhesion points after FAK blockage. I have then studied the regulation of actin cytoskeleton on eNOS signaling. Treatment with pan- Rho-associated protein kinases (ROCK) inhibitor Y27632 not only reorganized actin filaments distribution (data not shown) but significantly enhanced eNOS expression level in both HUVECs cultured on Col. I and BOECs on POLY. ROCK consists of two isoforms, namely ROCK1 and ROCK2, which exert different function on actin cytoskeleton with ROCK1 on actin destabilization and ROCK2 on actin stabilization and polymerization [254]. A previous study has demonstrated that ROCK2, but not ROCK1, negatively regulates eNOS expression by phosphorylating eukaryotic elongation factor 1 alpha 1 (eEF1A1), which is required for binding to eNOS mRNA [255]. In line with previous finding, I have here demonstrated that inhibition of ROCK2, but not ROCK1, by siRNA transfection significantly enhance the eNOS level in BOECs similar to that treated with pan-ROCK inhibitor Y27632. Furthermore, treatment with the specific inhibitor of actin polymerization Cyto D significantly enhanced eNOS level in BOECs. Taken together, these results suggest a negative regulation of actin polymerization on eNOS expression induced by ECM substrates. In line with our findings, previous study has demonstrated that mice treated with a Rho inhibitor or the actin cytoskeleton disrupter Cyto D showed increased vascular eNOS expression and activity, and these changes were associated with a decrease in cerebral infarction size after middle cerebral artery occlusion [184]. The regulation of eNOS by ROCK has been summarized in Figure 28.

It is noticed that different culture period was used when testing the effects of Col. I, β 1 integrin, FAK, and ROCK inhibitors on eNOS signaling in HUVECs and BOECs. The

main cause is the difference between cell-deposited matrix and matrix coating. Cell-deposited matrix not only contain different amount but conformation compared to external matrix coating and as such may result in significant difference for intracellular regulation. To better compare the regulation of matrix on eNOS signaling in both cells, one improved method maybe made by culturing HUVECs on BOECs or collagen I siRNA BOECs deposited matrix and study the effect.

For graft endothelization, the activity of eNOS is very critical as this enzyme regulates anti-thrombosis and inhibits platelet aggregation and smooth muscle cell proliferation. However, under pathophysiological conditions, eNOS is uncoupled and produces superoxide instead of NO which detrimentally affects EPC functions [256-258]. Thum and colleagues have isolated EPCs from healthy and diabetic patients and demonstrated that the excessive $O_2^- \cdot$ production, reduced intracellular BH_4 concentration, as well as reduced eNOS monomer/eNOS dimer ratio in diabetic patients reduced the number of EPCs and endothelial CFUs [258].

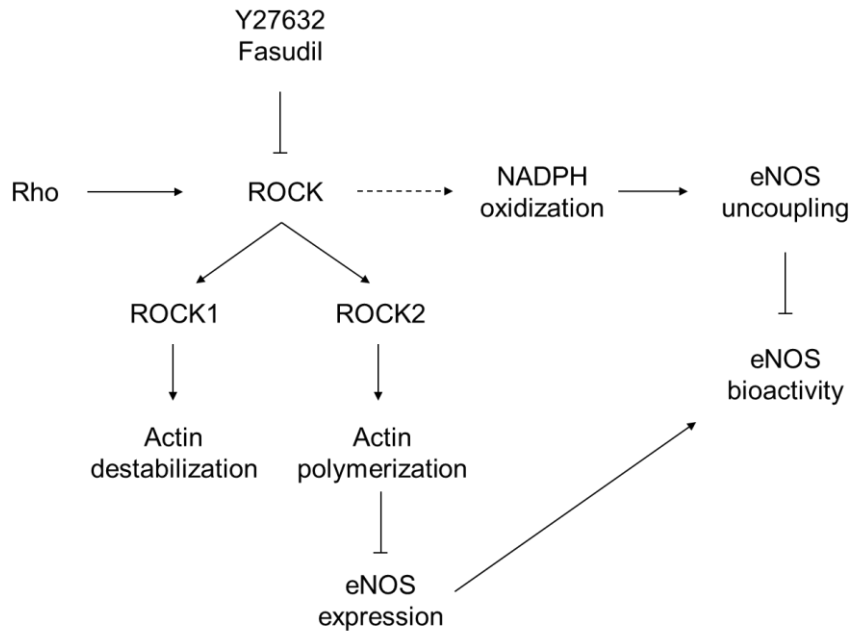


Figure 28: The role of ROCK on eNOS regulation. Rho A, B, and C activates ROCK and subsequently either induces actin destabilization or polymerization which are regulated by ROCK1 or ROCK2, respectively. Polymerized actin through ROCK2 further reduces eNOS expression. The activation of ROCK may induce NADPH oxidization and further eNOS uncoupling. Both reduced eNOS expression and increased eNOS uncoupling decrease eNOS bioactivity.

I have measured the basal nitrotyrosine levels in BOECs and HUVECs and found that the nitrosylation level in BOECs is significantly higher than that in HUVECs. Either BH4 (eNOS uncoupling inhibitor) or SOD (superoxide scavenger) reduces the generation of nitrotyrosine suggesting that the increased nitrotyrosine is at least partially due to eNOS uncoupling and increased superoxide anion. Interestingly, ROCK inhibition by Y27632

reduces the superoxide production and nitrotyrosine expression level in BOECs. Previous study has demonstrated that treatment with Y27632 blocked the arsenic trioxide-induced NADPH oxidation in human macrophages [259]. This study suggests that Y27632 induced nitrotyrosine decrease in BOECs may be due to inhibition of NADPH oxidation as BOECs were cultured from monocyte population and may contain similar biological behaviors. Further investigation should focus on NADPH oxidation and eNOS uncoupling regulation by ROCK inhibition. Taken together, the results in Chapter 4 demonstrated that Col. I induced eNOS decrease is through a β 1 integrin/FAK/actin cytoskeleton polymerization dependent mechanism. Furthermore, ROCK inhibition not only increases eNOS expression but bioactivity levels in BOECs which provide a new drug candidate to improve functional endothelialization.

CHAPTER 5: PERSPECTIVE

5.1. Summary

The investigations from Chapter 2 through Chapter 4 present three independent but highly related studies that describe biological role of eNOS in endothelial cells, especially in BOECs, from different aspects. In Chapter 2, I have demonstrated that eNOS transfection improved in vitro therapeutic effect such as migration, network formation, and attachment and spreading on ECM substrates, in BOECs and confirm the critical role of eNOS in these cells. However, the endogenous eNOS expression/activity level was found markedly lower in BOECs compared to mature ECs. In Chapter 3, I have demonstrated that higher Col. I expression and deposition in BOECs compared to HUVECs and HAECs results in lower basal eNOS expression. ECM substrates regulate signaling transduction through integrin, focal adhesion, and actin cytoskeleton. In Chapter 4, I have demonstrated that the regulation of eNOS in BOECs and HUVECs by ECM substrates is through a $\beta 1$ integrin/FAK/actin polymerization dependent mechanism. These three studies have provided significant contributions for better understanding eNOS biology in endothelium, development of eNOS and BOECs for endothelialisation of cardiovascular biomaterials, and improvement of BOECs phenotype as a therapy for cardiovascular diseases. The schematic signaling pathway is shown in figure 29.

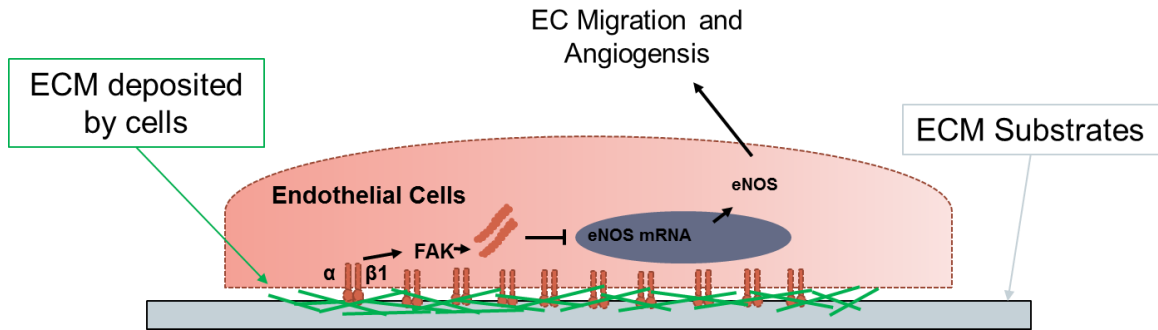


Figure 29: Schematic depicting eNOS regulation by ECM substrates in BOECs and HUVECs. This thesis has shown that in both BOECs and HUVECs, either ECM deposited by cells or ECM substrates supplied externally negatively regulates eNOS gene and protein expressions through a $\beta 1$ integrin/FAK/actin polymerization dependent mechanism. The enhanced eNOS expression in either ECs or EPCs plays an important role in cell migration, network formation, and adhesion to ECM substrates.

5.2. Understanding the regulation of eNOS by integrin and focal adhesion complex

Endothelial NOS, a key enzyme constitutively expressed in endothelium that catalyzes the production of NO, regulates many angioprotective mechanisms such as vessel tone, neovascularization, anti-thrombosis, and inflammation under physiological conditions and can be regulated by many physiological and pathophysiological cues (reviewed in [152]). Hemodynamic shear stresses not only provide directional force but exert fundamental effect on eNOS signaling in endothelium. This mechanotransduction was mainly regulated through cell-matrix interactions which involve integrin binding and

integrin associated protein activations. However, very few studies have investigated the regulation of ECM substrates on eNOS signaling in endothelial cells [180-182]. I have demonstrated in Chapter 3 that after 3 days culture on Col. I, FN, or LAMA, the eNOS protein and gene expressions in HUVECs was significantly reduced compared to control, suggesting a strong matrix mediated eNOS regulation in ECs. Furthermore, the expression level of eNOS is similar when HUVECs were cultured on different matrix proteins, suggesting that eNOS regulation by ECM substrates is component-independent. Cell adhesion to ECM substrates is regulated through binding of transmembrane heterodimeric proteins integrin to corresponding ligands, which subsequently induces the formation of focal adhesion complexes. The nascent focal complex recruits multiple focal adhesion proteins such as ILK, vinculin and FAK to induce integrin clustering, assembly of a multiprotein complex and actin cytoskeleton assembly that is capable of linking integrins to the actin cytoskeleton and communicating with signaling pathways [7]. Therefore, I have investigated the role of integrin, focal adhesion proteins, and actin cytoskeleton on eNOS regulation and demonstrated in Chapter 4 that the blockage of $\beta 1$ integrin, FAK, or actin cytoskeleton polymerization inhibits the downregulation of eNOS expression in response to ECM substrates. This work has provided not only insights on eNOS regulation by ECM substrates, but guidance to further understand the biological behavior of hemodynamic shear stress on eNOS signaling in endothelium.

5.3. Development of eNOS-transfected BOECs for endothelialisation of cardiovascular biomaterials

Endothelium, an important regulator of vascular homeostasis, produces factors such as nitric oxide, prostacyclin to prevent thrombosis and inflammation under physiological conditions and has been widely used to coat the cardiovascular biomaterial surfaces [49-55]. However, a purified functional autologous endothelial cell population is hard to achieve. BOECs, cultured from blood mononuclear cells, are growing in cobblestone morphology, displaying contact inhibited growth, and expressing protein profiles similar to mature ECs and as such may be a promising cell candidate for endothelialisation of cardiovascular biomaterial surfaces [84]. Regenerated endothelium on denuded or implanted biomaterial surfaces often lack appropriate integrity and function, displaying poorly formed cell junctions and reduced expression of antithrombotic proteins [205]. Endothelial NOS regulates many angioprotective mechanisms and as such is an ideal candidate gene for restoring endothelial functional activity (reviewed in [152]). In Chapter 2, I have described the protocol to effectively derive large number of BOECs from leukapheresis product and studied the in vitro effect of enhanced eNOS expression in these cells. The novel findings in this chapter are listed below.

1. Over 10^9 BOECs with similar phenotype to mature ECs were obtained at passage 2 from a single leukapheresis product cultured under xenofree condition.
2. Minicircle DNA is superior to conventional plasmid to enhance transgene expression in BOECs.
3. Endothelial NOS transfection in BOECs results in increased cell migration, angiogenesis, and adhesion in vitro.

Leukapheresis is an established and well-tolerated clinical procedure that leads to fewer complications [217,218]. To my knowledge, I am the first to report the generation of BOECs from large-volume apheresis products although many other groups have demonstrated obtaining MACs or EC colonies; however, most of these studies used Granulocyte Colony-Stimulating Factor (G-CSF) pre-treatment in the donors [220,222,223]. Because G-CSF potently stimulates WBC production and depresses the angiogenic potency of EPCs, it may not be tolerated well in severely ill cardiovascular patients [224,225]. In my study, I have isolated and expanded BOECs from apheresis products without pre-treating the donors with G-CSF. This was performed under xenogene free conditions that allowed for more than a 200-fold expansion. Previous studies have utilized FBS for BOEC expansions [226] yet the use of animal products would be less desirable for the development of clinical materials. I have then demonstrated the effectiveness of minicircle eNOS transfection of BOECs. eNOS-transfected BOECs displayed markedly enhanced substrate adhesion and although eNOS expression reduced within days after transfection, it is likely that this transient response would be sufficient to establish the cells on vascular surface and mitigate initial post-implantation thrombotic response. I observed that the cytoskeleton of the BOECs was altered and more focal adhesion sites present with transfection. Enhanced cell adhesion may be related to increased cell deformability and spreading, leading to more cell-substrate contact points. Increases in cell adhesion and area may stimulate the rapid establishment of an endothelial monolayer. Thus, eNOS-transfected BOECs may be ideal for endothelial regeneration. One of the future experiments is to apply eNOS-transfected BOECs on OPN-coated vascular graft (e.g. ePTFE). The endothelialisation can be

measured in vitro by staining EC markers such as PECAM, VE-Cadherin, eNOS after several days of seeding. The cell spreading can be determined by cytoskeleton staining (e.g. Phalloidin). After cells reach confluency, eNOS-transfected BOECs seeded graft, scramble BOECs-seeded graft, and control graft will be implanted into diseased animals followed by assessment on graft patency and neointimal hyperplasia at the end of experiment to further determine the efficacy of eNOS-BOECs and OPN coating for graft endothelialization.

5.4. The development of BOECs as a therapy for cardiovascular diseases

EPCs are a subpopulation of hematopoietic cells originated from bone marrow and released into circulation with the potential to differentiate towards endothelial-like cells [66]. There are two subpopulations of EPCs cultured from blood mononuclear cells, MACs and BOECs. This BOECs displays similar phenotype with mature ECs, exert rapid proliferative capacity [101,201] and as such is an ideal in vitro model for endothelialization. Our group has demonstrated that the therapeutic capacity of MACs is markedly enhanced by transient transfection with human eNOS especially in models of PAH [199]. Some other studies have looked at this enzyme in BOECs [200,260]. For example, Kong and colleagues have transfected BOECs with eNOS and transplanted them into rabbits which were subjected to balloon angioplasty of common carotid artery. After two weeks, morphometric analysis revealed a significant decrease of neointima/media ratio in eNOS-transfected BOECs transplanted vessels compared to

BOECs transplanted vessels [200]. In line with previous findings, in Chapter 2, I have enhanced the eNOS expression level in BOECs by transient transfection and demonstrated increased cell migration and network formation due to transfection, suggesting the therapeutic potential of these genetically modified cells for cardiovascular diseases. However, I have shown in Chapter 3 that compared to mature endothelium, the endogenous eNOS expression/activity levels is lower but eNOS uncoupling is markedly higher in BOECs, which may be a huge drawback to functional vasculature formation. Previous studies have demonstrated that ECM proteins including Col. I, FN and LAMA reduce eNOS expression and NO production in mature endothelial cells, respectively [6-8], and a recent study has shown that BOECs deposit extensive amounts of Col. IV, FN, and LAMA [9]. I have shown in Chapter 3 that the markedly higher collagen I expression and deposition in BOECs compared to mature endothelial cells results in lower eNOS expression and this effect was regulated by a $\beta 1$ integrin/FAK/actin cytoskeleton polymerization dependent mechanisms. Thus, by either reducing collagen I expression/deposition, or blockage of cell-matrix interactions proteins (such as integrin, FAK, and actin cytoskeleton) will result in enhanced eNOS expression in BOECs, which may further improve the functionality of this cells for cardiovascular diseases treatment.

One of the explanations of differential eNOS expression/activity in BOECs may be due to the incubation with monocyte-secreted TGF- β for 2 – 3 weeks post-isolation. Study by Moonen et al have demonstrated that after stimulation with TGF β 1, BOECs lost expression of endothelial markers such as CD144, eNOS, vWF, and thrombomodulin and endothelial anti-thrombogenic function and simultaneously, mesenchymal markers such as α -SMA, SM22 α , SMMHC, and calponin expression were gained, cytoskeletal

rearrangements occurred, and cells acquired a contractile phenotype [261]. Although further comprehensive studies (e.g. microarray) need to be performed to confirm this hypothesis, TGF β 1 inhibitor such as suramin sodium or GW788388 may be added into the co-culture system to potentially inhibit the loss of eNOS expression/activity and promote functions in BOECs.

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