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Vascular Protective Effects of Netrin-1 and Netrin-1 Pre-conditioned Endothelial Progenitor Cells

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Vascular Protective Effects of Netrin-1 and Netrin-1 Pre-conditioned Endothelial Progenitor Cells

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Molecular Cellular and Integrative Physiology

by

Meng-Chia Liu

2018

ABSTRACT OF THE DISSERTATION

Vascular Protective Effects of Netrin-1
and Netrin-1 Pre-conditioned Endothelial Progenitor Cells

by

Meng-Chia Liu
Doctor of Philosophy in Molecular Cellular and Integrative Physiology
University of California, Los Angeles, 2018
Professor Hua Cai, Chair

Many of the pathophysiological processes of vascular disease are attributed to endothelial dysfunction. Under normal conditions, nitric oxide ($NO^{\bullet}$) produced by endothelial $NO^{\bullet}$ synthase (eNOS) plays a major role in maintaining vascular homeostasis. Due to a damage on endothelial cells (ECs), smooth muscle cells (SMCs) are proliferated and migrated to form neointimal cusion that can occlude blood vessels due to reduced $NO^{\bullet}$ bioavailability. Platelets are also aggregated and activated to promote extracellular matrix production as well as additional inflammatory responses in the environment of disturbed $NO^{\bullet}$ production. Therefore, one of the most effective approaches to protect the vasculature from these pathological circumstances would be to improve endothelial function. Cell-based therapies, such as approaches utilizing endothelial progenitor cells (EPCs), aim at accelerating revascularization. However, the challenge in cell isolation and acquisition of

sufficient cell number of EPCs from circulation has limited its clinical application. Nonetheless, our studies have demonstrated pre-conditioning of EPCs with netrin-1 can markedly improve their functions as to improved proliferative activity and survival capacity, and that with this treatment much less number of EPCs is sufficient to induce robust vascular protection when delivered in vivo.

In our previous study, we demonstrated that $NO^{\bullet}$ mediates mitogenic effects of netrin-1 on ECs via activation of DCC/ERK1/2 axis. The current study for the first time further revealed promoted functions of EPCs by netrin-1, which is also dependent on DCC and $NO^{\bullet}$ production, resulting in protection against vascular endothelial damage. For these studies we employed two animal models; femoral artery wire injury model and apoE knockout mice fed high fat diet, for the development of neointima and atherosclerosis respectively. In addition to demonstration that netrin-1 protein infusion itself is robustly protective in these models, delivery of netrin-1 pre-conditioned EPCs proved to be markedly beneficial via augmented proliferation and resistance to oxidative stress. Moreover, the signaling pathway of $NO^{\bullet}$/cGMP/p38 MAPK was identified to mediate netrin-1 inhibition of SMC migration. The inhibitory effect of netrin-1 on monocytes adhesion to EC through UNC5B is also involved in the protection against atherogenesis. Although further studies are

required, the present study indicates the strong potential of netrin-1 and netrin-1 pre-conditioned EPCs in vascular protection.

The thesis of Meng-Chia Liu is approved.

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University of California, Los Angeles

2018

Dedicated

To my father, Kinchu Ryu,
who made me dream of being a scientist
by showing me what is it like to have a lifework
that wonders you and colors your life forever.
I hope you rest in peace and proudly watching me from heaven.

To my mother, Hinhei Sai-Ryu,
who always puts me in priority among so many other things,
with a strong and gentle soul.

To my siblings, Yuri Nakano and Masao Ryu,
for being my best friends as well as role models.

You have always believed in me and supported me with unconditional love.
This work is a proof of receipt of your support, with so much love!

Norika

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List of Acronyms

EC	Endothelial cell
SMC	smooth muscle cell
VSMC	vascular smooth muscle cell
$NO^{\bullet}$	nitric oxide
ROS	reactive oxygen species
eNOS	endothelial nitric oxide synthase
NADPH	nicotinamide-adenine-dinucleotide phosphate
BH_4	tetrahydrobiopterin
sGC	soluble guanylyl cyclase
cGMP	cyclic guanosine monophosphate
MAPK	mitogen activated protein kinases
VEGF	vascular endothelial growth factor
iNOS	inducible nitric oxide synthase
EPC	endothelial progenitor cell
HSC	hematopoietic stem cell
VEGFR2	VEGF receptor-2
BM	Bone marrow
VCAM-1	vascular cell adhesion molecule 1
SOD	superoxide dismutase
EGF	epidermal growth factor
DCC	deleted in colorectal cancer
DES	drug eluting stents
PTIO	2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
vWF	von Willebrand Factor
ESR	electron spin resonance
BAEC	bovine aortic endothelial cell
WT	wildtype
EBM-2	endothelial growth basal medium-2

H_2O_2	hydrogen peroxide
apoE	apolipoprotein E
LDL	low density lipoprotein
Ox-LDL	oxidized- low density lipoprotein
ER	endoplasmic reticulum
DAPI	4’6-Diamidino-2-Phenylindole, Dihydrochloride
HFD	high fat diet
HDL	high density lipoprotein
CTRL	control
Rb	retinoblastoma
CDK	cyclin dependent kinase
CKI	cyclin dependent kinase inhibitor

Acknowledgments

I feel very privileged to have been given an opportunity to have Professor Linda Cai as my adviser, who is intelligent, thoughtful, and extremely talented and successful scientist. While I was pursuing a master's degree in Japan, I had a chance to work in her laboratory, and that was when she encouraged me to proceed to Ph.D. program at UCLA. I had never even dreamed to pursue doctoral degree from UCLA at that time. However, the belief she had in me changed my life. Professor Cai directed me to efficiently design a study with a great impact. She often spent hours discussing my research progress regardless of day or night. She guided me to be a better scientist, but nonetheless, she gave me plenty of support mentally as well. She is my role model as a researcher, and I also respect her as a person who is always cheerful, kind, and hard working.

I would like to express my appreciation to the other members of my Ph.D. thesis committee. They gave me insightful advice on my research since the oral qualifying exam that I had in my second year. Besides their role in my research, they were very encouraging all the time. Professor Yibin Wang supported me even before the Ph.D. program starts. I am extremely grateful to him. Professor Tzung Hsiai was always interested in my research and that encouraged me to strive to work on my research even more. Professor Atsushi Nakano always gave me keen opinions on my research with a humble attitude, and they brushed up our studies. Professor Wang, Professor Hsiai, and Professor Nakano helped me build my knowledge and skills to make significant progress in my research. I feel very honored to get to know them, who I admire so much.

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Biographical sketch

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[Peer Reviewed Publications]

1. **Liu NM,** Minamisawa S. Unique Phenotypes of Endothelial Cells in Developing Arteries: A Lesson from the Ductus Arteriosus. ***InTech (Open access book, chapter 10)*** April 2017
2. Zhang Y, **Liu NM**, Wang Y, Youn JY, Cai H. Endothelial Cell Calpain as a Critical Modulator of Angiogenesis. ***Biochimica et Biophysica Acta***. Mar 2017;1863(6):1326-1335
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4. **Liu NM**, Yokota T, Maekawa S, Lü P, Tei I, Taniguchi H, Yokoyama U, Kato T, Minamisawa S. Transcription Profiles of Endothelial Cells in the Rat Ductus Arteriosus during a Perinatal Period. (2013) ***PLoS One***. Sep 27;8(9):e73685
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1. Introduction

1-1. Endothelial Dysfunction in Cardiovascular Diseases

Endothelial cells (ECs) tightly connect to adjacent cells and form a single layer to cover the inner surface of vasculature in the entire body. They are making the first contact to circulating blood for vessels to regulate their homeostasis (Summarized in Figure 1-1). The ECs play a crucial role in controlling vascular tone, permeability, attraction of blood cells including lymphocytes that exhibit immunity, and proliferation/migration of underlying cells such as pericytes and smooth muscle cells (SMCs). Of note, nitric oxide ($NO^{\bullet}$) that generated from ECs has been long believed to play the central role in maintaining vascular homeostasis. Therefore, endothelial dysfunction initiates vascular occlusion via proliferation of SMC and accumulation of extracellular matrix, as well as recruitment of lymphocytes. Moreover, dysfunctional ECs retain oxidized lipoprotein in the subendothelial space. This further activates ECs and recruits monocytes which differentiate into macrophages and further mediate the formation of foam cells. Once foam cells are formed, there would be even more recruitment of inflammatory cells and malignant type of lipoprotein modification and thus build up lesions rapidly. These events are major components of atherogenesis, which

represents an inflamed state of artery. While normal ECs construct a barrier, which integrity is maintained via production of $NO^{\bullet}$ that reduces cellular oxidative stress [1]. Major cardiovascular risk factors such as aging, smoking, hypertension, hypercholesterolemia and hyperglycemia are known to alter endothelial physiology that leads to endothelial dysfunction. Importantly, in the presence of these cardiovascular risk factors, reactive oxygen species (ROS) degrade $NO^{\bullet}$ rapidly and alter the normal function of ECs, consequently leading to pathophysiological responses. Hence, repairing abnormality in EC physiology has a great potential to stop the progression of cardiovascular diseases. It is also important to develop methods to assess EC pathophysiology for early diagnosis, which can be utilized to minimize adverse events later in patients.

Figure 1-1.

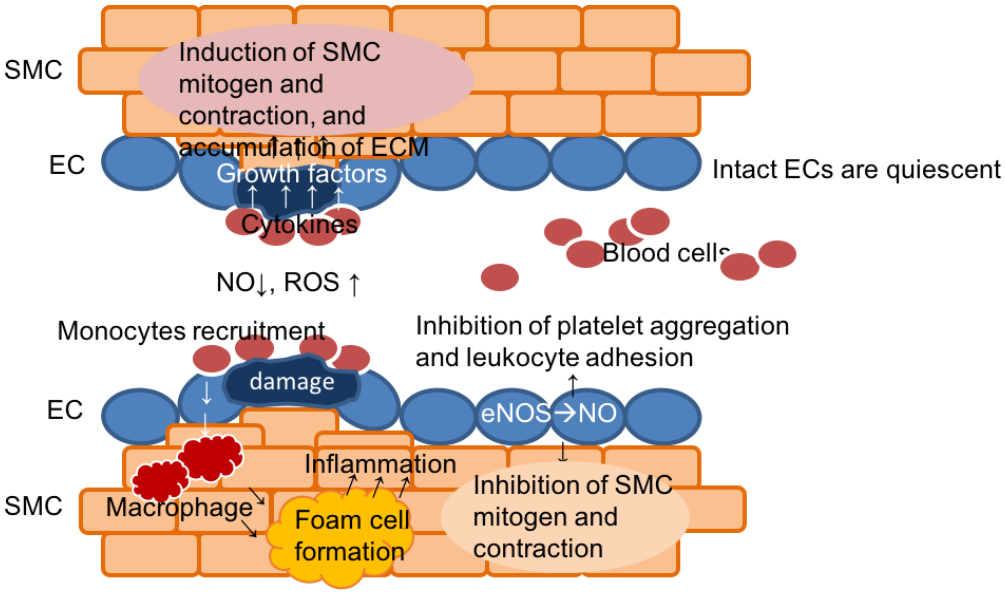

Figure 1-1. A central role of endothelial cells in regulating vascular homeostasis. The lining of healthy ECs constantly produce $NO^{\bullet}$, which inhibits platelet and monocyte recruitment as well as SMC migration and proliferation, thus maintaining vascular relaxation. Damaged ECs are shown in dark blue. When endothelium is dysfunctional, there is a reduction in $NO^{\bullet}$ bioavailability often consequent to an increased production of ROS, which leads to platelet aggregation or leukocyte adhesion to the intima. Cytokines produced from lymphocytes induce growth factor production and cause SMC hyperplasia and contraction. Monocyte recruitment due to reduced $NO^{\bullet}$ bioavailability leads foam cell formation in the subendothelial domain and triggers more inflammatory responses.

This figure is modified from *N Liu et at. Chapter 10, InTech (2017)*

1-2. Nitric Oxide and Oxidative Stress in Endothelial Cells

Disturbed production and reduced bioavailability of $NO^{\bullet}$ are major features of endothelial dysfunction. $NO^{\bullet}$ is generated from the amino acid L-arginine via activation of the enzyme endothelial NO synthase (eNOS), in the presence of other co-factors such as nicotinamide-adenine-dinucleotide phos-phate (NADPH) and tetrahydrobiopterin (BH_4) [2]. Although $NO^{\bullet}$ has a short half-life approximately up to 6 to 30 second s[3], it is continuously synthesized in ECs and diffuses to adjacent cells. When $NO^{\bullet}$ is reached into SMCs, proliferation and migration are inhibited by activating soluble guanylyl cyclase (sGC) and sGC consequently produces cyclic guanosine monophosphate (cGMP). This is the most well-established pathway of $NO^{\bullet}$ regulating vasodilatation. ENOS mutant mice display hypertensive phenotype [4]. Activation of $NO^{\bullet}$-sGC-cGMP pathway also prevents platelet activation and its aggregation to endothelium. Recent studies have shown that platelets facilitates interactions between endothelium and inflammatory cells including monocytes, neutrophils and lymphocytes, largely via P-selectin[5]. Therefore, the inhibition of platelet aggregation by $NO^{\bullet}$ protects the blood vessels from inflammatory insults (Summarized in Figure 1-2).

A great deal of angiogenic signaling pathways via $NO^{\bullet}$ produced from eNOS have

been reported. The proliferation, migration and anti-apoptosis effects on ECs are mediated by sGC/cGMP pathway, as well as s-nitrosylation, a post-translational modification adding nitrogen monoxide group [6]. Various mitogen activated protein kinases (MAPKs), such as ERK, MEK and AKT, are activated by NO. $NO^{\bullet}$ also can induce expression of endogenous angiogenic factors, often represented by vascular endothelial growth factor (VEGF).

Therefore, at least in the context of pro-angiogenesis which is involved in the repair of ischemic diseases and wound healing, $NO^{\bullet}$, especially produced by eNOS in EC, is beneficial. However, there are some circumstances in which $NO^{\bullet}$ can be harmful. An inducible isoform of NOS, iNOS, is usually induced by inflammatory cytokines and/or ROS, and generates $NO^{\bullet}$ 100 to 1000 times more than eNOS does [7]. Such high concentration of $NO^{\bullet}$ produced by iNOS induction promotes DNA damage and drives vascular cells apoptotic. In tumor angiogenesis, iNOS is more dominant than other isotype of NOS. As the tumor progress its malignancy, iNOS induce apoptosis in normal cells and acquisition of resistance to apoptosis in tumor cells by clonal selection of adaptation [6].

Thus, maintaining $NO^{\bullet}$ bioavailability in harmony is indispensable for a healthy vasculature. The definition of a healthy vasculature is being supple, with no excessive intimal cushion and blood or inflammatory cell aggregation. To accomplish it, activating eNOS to

produce more $NO^{\bullet}$, as well as inhibiting oxidative stress, such as by preventing eNOS uncoupling, would be necessary. The dysfunctional EC should be replaced to a functioning EC, in a physiological manner. Resident adjacent ECs can contribute to replace the damaged EC by their proliferation. The breakdown of cell-cell junctions between damaged EC and its adjacent ECs would trigger release of intercellular growth and differentiation factor, so that the resident EC gets to be activated and proliferated [8]. However, with the presence of more cardiovascular risk factors, there would not be enough resident ECs that can proliferate to repair. A replenishment of damaged ECs by injecting exogenous endothelial progenitor cells (EPCs) is believed to be promising in future clinical therapy. The role of EPCs in vascular repair is stated in the next section.

Figure 1-2.

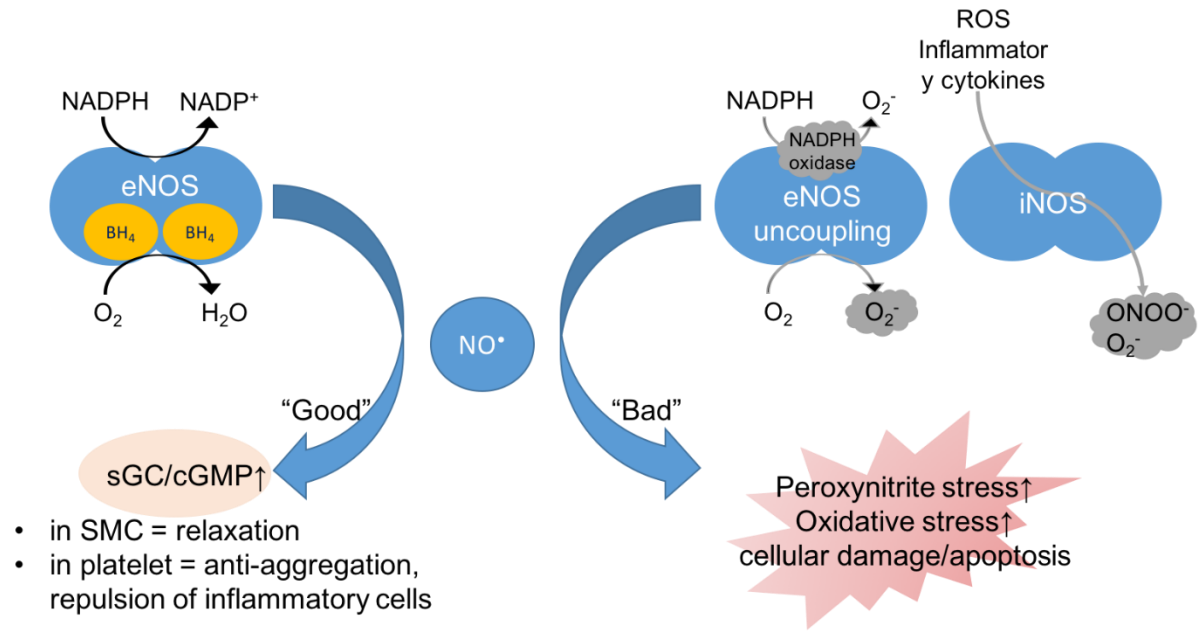

Figure 1-2. Differential roles of modest ("good") and high ("bad") levels of NO•. The "good" NO• produced from eNOS with its co-factor BH_4 induce sGC/cGMP in SMCs and platelet. Whereas the "bad" NO• generated by eNOS uncoupling or iNOS exacerbate oxidative stress and increase peroxynitrite formation to cause various cellular damages.

1-3. Endothelial Progenitor Cells in Vascular Repair

EPCs were first characterized by Asahara et al. in 1997[9].They can be defined by the common surface markers for hematopoietic stem cells (HSCs) and ECs, such as c-Kit, Sca-1, CD34, CD133, Tie-2 and VEGF receptor-2 (VEGFR2), confirming that EPCs are derived from hemangioblast and capable to differentiate into mature ECs. Bone marrow (BM) transplantation studies demonstrated that EPCs derived from BM contribute to both physiological and pathological neovascularization. The postnatal neovascularization utilizing BM-derived EPCs are seen during tumor growth, wound healing, ischemic muscle healing, and cornea micropocket angiogenesis after surgery, as well as in uterus endometrial formation[10]. These evidences suggest neovascularization can occur not exclusively by proliferating resident ECs but also by incorporating BM-derived EPCs. Those findings from BM transplantation also show that EPCs are mainly produced in BM, and mobilize into peripheral blood stream and then home to endothelium. Although not yet entirely revealed, the recruitment of circulating EPCs is mostly augmented through chemotaxis and adhesion mechanisms. The interaction of chemokine receptor CXCR-4,which is expressed on EPCs, and its ligand stromal cell-derived factor 1 (SDF-1, also known as chemokine-CXCL-12) is the most established pathway in EPC mobilization[11][12]. Other than this chemokine factor,

EPCs also express α4-integrins on the membrane and bind to vascular cell adhesion molecule 1 (VCAM-1), which is expressed on endothelial and stromal cells[13][14]. However, how these factors are regulating the downstream signaling to achieve the EPC homing is largely unknown. More recent studies reveled the involvement of c-kit phosphorylation in response to CXCR-4/SDF-1 and α4-integrins/VCAM-1 binding[15]. Investigation on detailed mechanisms of BM-derived EPC kinetics is hampered due to the lack of precise tracking method and incomplete characterization and definition. The role of EPCs in postnatal vascularization is summarized in Figure 1-3.

Studies have consistently shown that the number, migratory activity and functions of circulating EPCs are compromised in those who have high risk of cardiovascular diseases including coronary artery disease and diabetes[16][17][18]. These studies implied the importance of EPCs in cardiovascular disease diagnosis and prognosis. Despite the fact that the remained questions in EPC molecular biology, several clinical studies of cell therapy had been conducted soon after discovery of BM-derived EPCs [19][20], but still have not reached to satisfaction in clinical applications (Table 3 in ref [21]). Therefore, improved means of transplantation of EPCs are required in patients without causing potential complication. One of the major concerns in the effective EPC delivery deals with disturbance from oxidative

stress, which is increased in patients with cardiovascular diseases. Functions of EPCs are impaired in the presence of ROS [22][23]. Thus, even though the enough number of EPCs is injected to patients with cardiovascular diseases, cells would undergo apoptosis and reduce the probability of sufficient homing to the desired site to promote re-endothelialization. Moreover, such suboptimal microenvironment can cause worse secondary effects from inflammatory cytokines generated by EPC apoptosis. It is obvious that simply increasing the number of EPC for delivery cannot solve the problem. A great effort has been made to optimize the method of EPC administration (Table 4 in ref [21]), but further addressing the way to protect EPCs from oxidative stress is desperately required. Indeed, $NO^{\bullet}$ bioavailability and scavenging ROS by inactivating NADPH oxidase and by upregulating superoxide dismutase (SOD) maybe beneficial [24][25][26]. $NO^{\bullet}$ production from EPC itself is also proved to exert antioxidant effect and protect vascular from further endothelial injury [27]. Hence, refining the method of administration as well as pre-conditioning of EPCs to protect them from oxidative stress would be effective approaches to improve EPC-dependent therapeutic applications.

Figure 1-3.

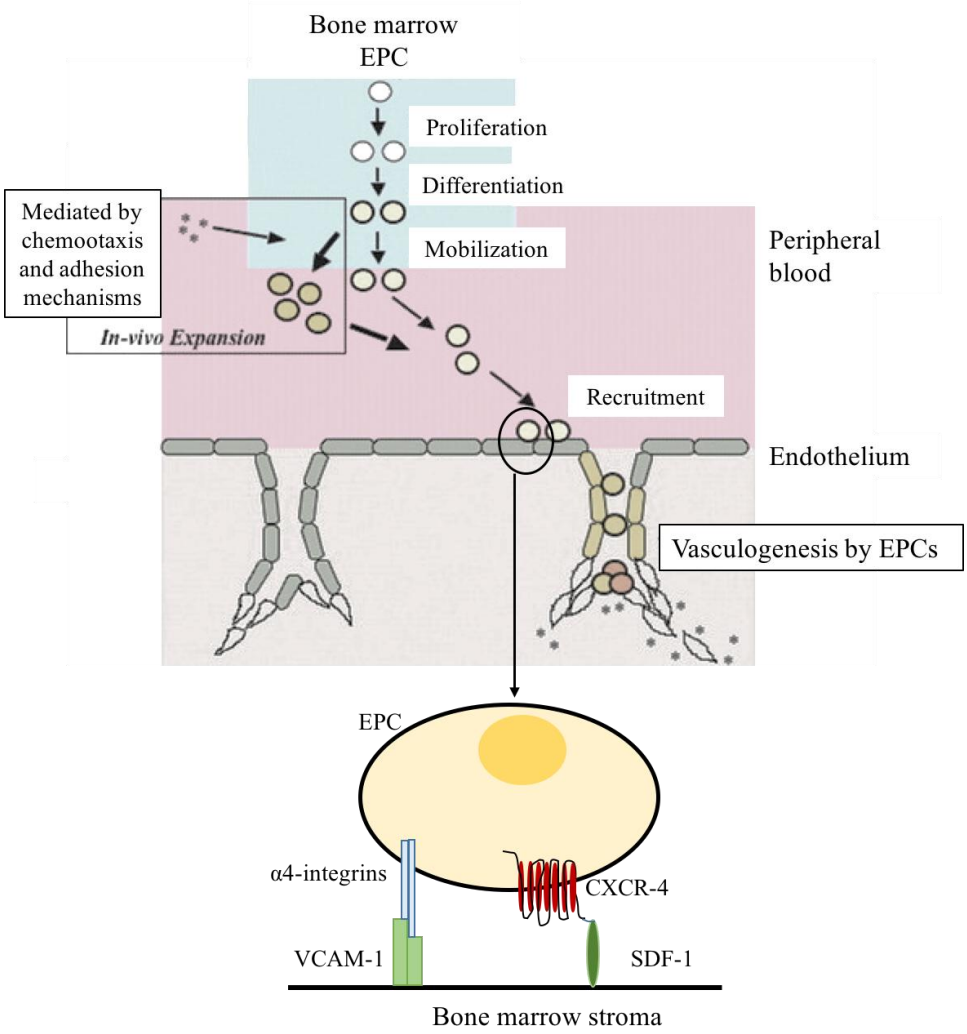

Figure 1-3. Neovascularization by EPC mobilization and recruitment. EPCs are derived from bone marrow and mobilize into peripheral blood circulation. Chemotaxis and/or adhesion molecules such as SDF-1 and VCAM-1 mediate EPC recruitment onto the endothelium to promote vasculogenesis.

The figure is modified from *Kawamoto A & Asahara T, Catheter Cardiovasc Interv. (2007)* and *Cheng M & Qin G, Prog Mol Biol Transl Sci. (2012)*

1-4. Endothelial Protective Effect of Netrin-1

Netrins were originally identified as a family of endogenous axon guidance molecule in 1990 by a genetic study using Caenorhabditis elegans [28]. Later netrins were found in other organisms and demonstrated to be secreted by floor plate cells, which promote circumferential spinal commissural axons during development [29][30]. Netrins are laminin related proteins in which N-terminal domains V and VI are homologous to each other[31]. The V domain in N-terminus consists of 3 epidermal growth factor (EGF) domains. Netrins-1, -2 and -3 are homologous to γ chain of laminin, whereas netrins-4, -G1 and G2 are homologous to β chain of laminin. Netrin-1 is the most extensively studied member of the family, and crystal structure studies of netrin-1 revealed that V and VI domains are responsible for its receptor binding [32][33]. There are two major receptors of netrin-1, Deleted in Colorectal Cancer (DCC) and UNC5B from UNC-5 family proteins. DCC and UNC5B are found to show an opposed response in cell migration, attractive and repulsive, to the concentration of netrin-1. Other than such biphasic chemotactic mechanism, DCC and UNC5B are known to be dependence receptors, meaning that unbound receptors could induce apoptosis instead of being quiescent and exhibiting pro-apoptotic effect in the presence of ligand netrin-1 [34].These features have made netrin-1 a unique molecule among other axon

guiding cues, such as ephrins, semaphorins and slits. Netrin-1 have been also found in various tissues outside of the central nervous system such as pancreas, mammary gland, lung, muscle, and vasculature [35]. Our studies have been focusing on the effect of netrin-1 in the cardiovascular system, specifically endothelial cells. (Netrin-1 and its receptor structures: see Figure 1-4)

The importance of netrin-1 in vascular cells began to take notice because of the findings on common molecular mechanisms between vascular and neural network formations. Not only the structure formation of lamellipodia and filopodia at axonal growth cones and endothelial tip cells are similar, but later it was found that they share molecular cues for growth guidance as well[36].While three other axon guidance cues ephrin, semaphorin and slit were demonstrated to have angiogenic activity one after another, the angiogenic role of netrin-1was also revealed in 2004[37][38]. Subsequently, our group showed this role of netrin-1 was mediated by the dominant expression of DCC in aortic endothelial cells and consequent activation of the mitogen activated protein kinase ERK1/2 and $NO^{\bullet}$ production via eNOS phosphorylation (Figure 1-5) [39]. As mentioned above, since $NO^{\bullet}$ production in endothelial cells is known to have mitogenic effect on endothelial cells, we speculated that netrin-1 can be a strong therapeutic candidate in cardiovascular diseases by restoring

endothelial dysfunction. Our group and others have published a substantial number of studies demonstrating the protective effect of netrin-1 on cardiovascular systems thus far [40][41][42][43], which suggests the promising utilization of netrin-1 in clinical therapy.

Figure 1-4.

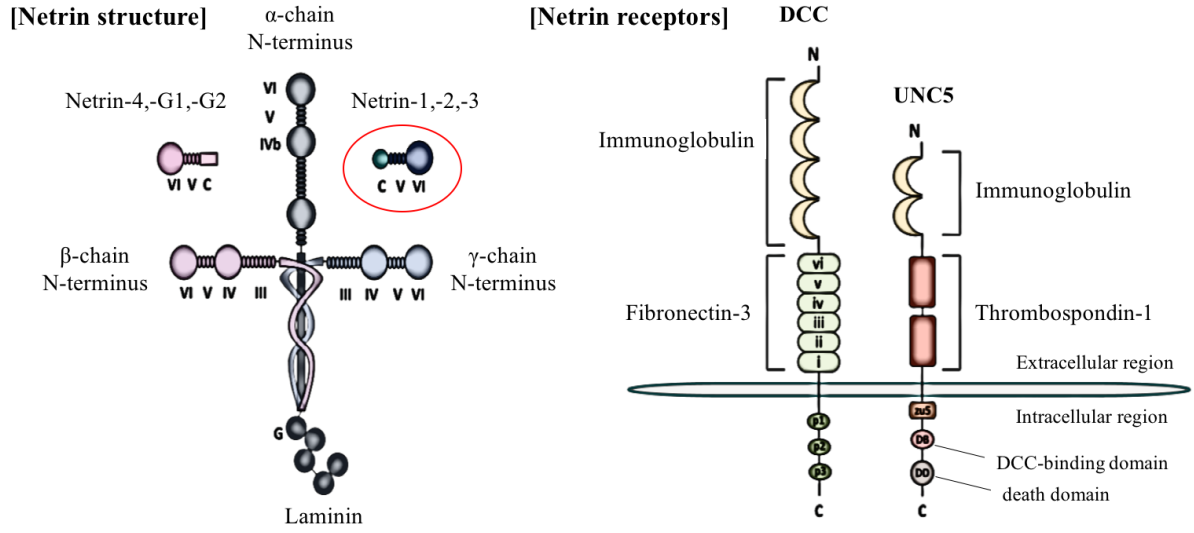

Figure 1-4. The structure of netrins and netrin receptors. Netrins are laminin related molecule that consist of three domains, V, VI, and C. Note that netrin-1 is homologous to the γ-chain of laminin N-terminus. Representative receptors, DCC and UNC5, are single-ass transmembrane proteins. Netrin-1 binds to extracellular domain of these receptors.

Figure is adopted from *Ko SY et al, Trends Mol Med. (2012)*

Figure 1-5.

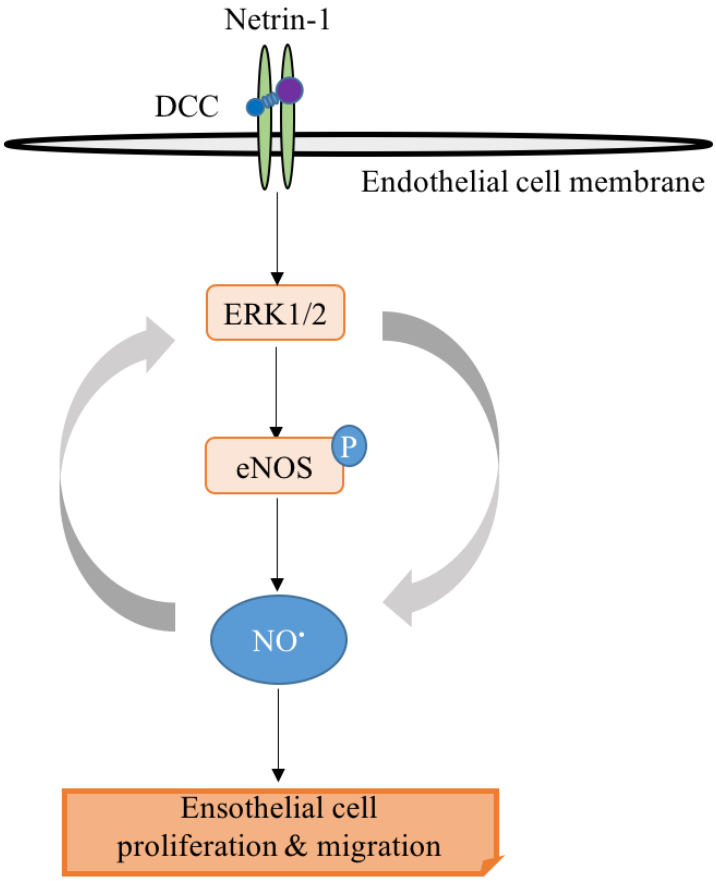

Figure 1-5. Netrin-1 induces angiogenesis via DCC/ERK1/2/eNOS/ $NO^{\bullet}$ signaling pathway. Netrin-1 binding to DCC activates ERK1/2, and subsequently eNOS phosphorylation at serine 1179 to produce $NO^{\bullet}$. Further, $NO^{\bullet}$ activate ERK1/2 to form a feed-forward cycle. This signaling cascade induces endothelial mitogenic responses.

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2. Vascular protective effect of netrin-1

As stated in the last section of the previous chapter, the protective effect of netrin-1 has gained a solid amount of evidence. In the following part of this chapter, the article "Attenuation of neointimal formation with netrin-1 and netrin-1 preconditioned endothelial progenitor cells", published in Journal of Molecular Medicine (March 2017, Volume 95, Issue 3, pp 335–348), with some of additional supporting information is presented. In this issue, we addressed more direct molecular mechanisms of netrin-1 in vascular endothelial recovery using femoral artery wire injury model mice to simulate restenosis followed by endothelial injury (Figure 2-1). The article also investigated the effect of netrin-1 on EPC function for the first time.

Figure 2-1.

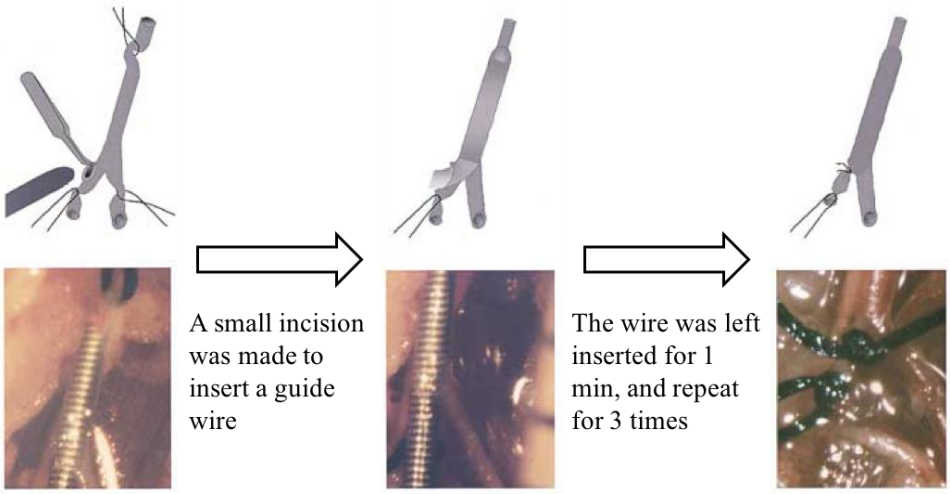

Figure 2-1. Femoral artery wire injury model. A guide wire was inserted from a small incision at the branch between the rectus femoris and vastus medialis muscles. Then the wire was left inserted for 1 min, 3 times to denude endothelial cells. The wound is closed by suturing.

This figure is modified from *Sata M et al, J Mol Cell Cardiol. (2000)*

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ORIGINAL ARTICLE

Attenuation of neointimal formation with netrin-1 and netrin-1 preconditioned endothelial progenitor cells

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Abstract
Restenosis after angioplasty is a serious clinical problem that can result in re-occlusion of the coronary artery. Although current drug-eluting stents have proved to be more effective in reducing restenosis, they have drawbacks of inhibiting re-endothelialization to promote thrombosis. New treatment options are in urgent need. We have shown that netrin-1, an axon-guiding protein, promotes angiogenesis and cardioprotection via production of nitric oxide (NO). The present study examined whether and how netrin-1 attenuates neointimal formation in a femoral wire injury model. Infusion of netrin-1 into C57BL/6 mice markedly attenuated neointimal formation following wire injury of femoral arteries, measured by intimal to media ratio (from 1.94 ± 0.55 to 0.45 ± 0.86 at 4 weeks). Proliferation of VSMC in situ was largely reduced. This protective effect was absent in DCC$^{+/-}$ animals. NO production was increased by netrin-1 in both intact and injured femoral arteries, indicating netrin-1 stimulation of endogenous NO production from intact endothelium and remaining endothelial cells post-injury. VSMC migration was abrogated by netrin-1 via a NO/cGMP/p38 MAPK pathway, while timely EPC homing was induced. Injection of netrin-1 preconditioned wild-type EPCs, but not EPCs of DCC$^{+/-}$ animals, substantially attenuated neointimal formation. EPC proliferation, NO production, and resistance to oxidative stress induced apoptosis were augmented by netrin-1 treatment. In conclusion, our data for the first time demonstrate that netrin-1 is highly effective in reducing neointimal formation following vascular endothelial injury, which is dependent on DCC, and attributed to inhibition of VSMC proliferation and migration, as well as improved EPC function. These data may support usage of netrin-1 and netrin-1 preconditioned EPCs as novel therapies for post angioplasty restenosis.

Key message

- Netrin-1 attenuates neointimal formation following post endothelial injury via DCC and NO.
- Netrin-1 inhibits VSMC proliferation in situ following endothelial injury.
- Netrin-1 inhibits VSMC migration via a NO/cGMP/p38 MAPK pathway.
- Netrin-1 augments proliferation of endothelial progenitor cells (EPCs) and EPC eNOS/NO activation.
- Netrin-1 enhances resistance of EPCs to oxidative stress, improving re-endothelialization following injury.

Keywords Netrin-1 · Neointimal formation · Vascular smooth muscle cell (VSMC) proliferation · VSMC migration · Femoral wire injury · Deleted in colorectal cancer (DCC) · Endothelial progenitor cells (EPCs) · EPC proliferation · Endothelial nitric oxide synthase (eNOS) phosphorylation · NO production · EPC resistance to oxidative stress · EPC homing · Re-endothelialization

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2-1. Introduction

Restenosis is a serious clinical problem as an untoward result of angioplasty, which is commonly performed in patients with myocardial infarction or severely occluded coronary artery. It is a major complication after angioplasty treatment of myocardial infarction. In order to prevent occurrence of restenosis/ re-occlusion of the coronary artery, different kinds of drug eluting stents (DES) have been developed. Although DES reduced the occurrence of in-stent restenosis compared with bare-metal stents, it raises the concern that most of drugs used in DESs inhibit re-endothelialization to promote thrombosis [1], which can be associated with more mortality. Hence, novel therapeutic strategies are in urgent need to prevent cardiovascular complications derived from restenosis.

The reason that restenosis remains unsolved clinically is likely attributed to its intricate mechanisms. Restenosis is primarily triggered by the damage of the endothelium that induces inflammatory response and thrombosis, followed by vascular smooth muscle cell (VSMC) proliferation and migration, and extensive vascular remodeling [2]. The endothelium has a crucial role in regulating vascular tone and structure by synthesizing nitric oxide ($NO^{\bullet}$) from endothelial NO synthase (eNOS), which is known to be a vasodilator with antiinflammatory and anti-thrombotic properties. $NO^{\bullet}$ produced in the endothelium activates

cyclic GMP (cGMP) and cGMP-dependent protein kinase in VSMC, which induce vasodilatation as well as inhibition of VSMC proliferation and migration. Therefore, augmentation of $NO^{\bullet}$ action at physiological levels may have good potential for attenuating restenosis. Nonetheless, none of the currently available therapies could ensure modest and continuous release of $NO^{\bullet}$. For example, nitroglycerin releases $NO^{\bullet}$ quickly but transiently to relieve chest pain associated with angina. Overdosing, however, leads to side effects such as severe hypotension [3]. In addition, it has been shown that many patients develop nitrate tolerance to become insensitive to the treatment [4].

Previously, our group have shown that netrin-1, originally discovered as an axon-guiding molecule [5, 6], exerts potent angiogenic and cardioprotective effects via the production of $NO^{\bullet}$, mediated by DCC/ERK1/2 dependent activation of eNOS [7–9]. Binding of netrin-1 to DCC activates ERK1/2 to produce $NO^{\bullet}$ by phosphorylation of eNOS at serine 1179 residue [8]. Further, production of $NO^{\bullet}$ in turn activates DCC or ERK1/2 pathway, resulting in a feed-forward mechanism to contribute to cardioprotective or angiogenic signaling [7–13]. Therefore, it is reasonable to hypothesize that netrin-1 may prevent restenosis following endothelial injury, via the protective effect of $NO^{\bullet}$.

In the present study, we examined the effects of netrin-1 on neointimal formation using a femoral artery wire injury model that simulates angioplasty procedure [14]. Netrin-1 infusion into femoral artery injured mice markedly attenuated neointimal formation in a DCC/$NO^{\bullet}$-dependent manner. VSMCproliferation in situ was reduced. We further demonstrated an inhibitory effect of netrin-1 on VSMC migration, via a $NO^{\bullet}$/cGMP/p38 MAPK pathway. In addition, netrin-1 infusion accelerated timely EPC homing after injury. Injection of netrin-1 preconditioned wild-type EPCs, but not EPCs of $DCC^{+/-}$ animals, largely abrogated neointimal formation. Proliferation, $NO^{\bullet}$ production, and resistance against oxidative stress-induced apoptosis of EPCs were significantly augmented by netrin-1. Taken together, our data for the first time establish that netrin-1 can serve as a potent vascular protective agent against restenosis after endothelial damage, and its effects are mediated by $NO^{\bullet}$-dependent inhibition of VSMC migration and augmentation of EPC functions.

2-2. Methods

Animals

All animal procedures were approved by the Institutional Animal Care and Usage Committee at the University of California, Los Angeles. Six- to eight-week-old male C57BL/6 mice from Charles River Laboratories (Wilmington, MA) were used. Animals were housed in a pathogen free facility, with food and water ad labium, and under a 12-h light/dark cycle.

Reagents

Netrin-1 (#1109-N1) was purchased from R&D Systems (Minneapolis, MN). Antibodies of proliferating cell nuclear antigen (PCNA/#sc-56) and Flk-1 (#sc-19530) were purchased from Santa Cruz (Dallas, TX). CD34 antibody (#ab8158) and GFP antibody (#ab290) were obtained from Abcam (Cambridge, MA), and Alexa Fluor 594 conjugateIsolectin GS-IB4 antibody (#I21413) was purchased from Life Technologies (Carlsbad, CA). CD133 antibody (#orb10288) was obtained from Biorbyt (San Francisco, CA). P38 MAPK inhibitor SB202190 (#559388) and JNK inhibitor SP600125 (#420119) were purchased from EMD Millipore (Germany). ERK1/2 inhibitor U0126 (#U120), cGMP antagonist Rp-8-Br-PET-

cGMPs (#B6684), NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO), anti-von Willebrand Factor (vWF) antibody (#F3520), and anti-actin antibody (#A2066) were all purchased from Sigma. Antibody for p38 MAPK (#506123) was obtained from Calbiochem. Antibodies for phospho-p38 MAPK (#9211), alpha-Tublin (#2125), eNOS (#9586), and phospho-eNOS (Ser1179) (#9571) were all purchased from Cell Signaling Technology (Danvers, MA). Goat anti-rabbit IgG-HRP conjugate (#172–1019) was obtained from Bio-Rad (Irvine, CA). Other chemicals were obtained from Sigma in the highest purity.

Surgical procedures—osmotic pump

Animals were anesthetized in a closed chamber filled with isoflurane, then quickly moved to a breathing mask supplied with 95%:5% O_2/CO_2 mixed with 1.5% isoflurane. Hair between the shoulder blades was removed using a hair removal lotion (Nair, Princeton, NJ), then disinfected using an iodine solution. A small incision was made in this area and a 4-week release osmotic pump (Durect Corp) was inserted into the left flank. Pumps were filled with either vehicle or netrin-1 (15 ng/ day, 1109-N1, R&D Systems, Minneapolis, MN). Femoral wire injury was performed a day after the insertion of the osmotic pump to allow for the pretreatment of netrin-1.

Surgical procedures—femoral wire injury model

Animals were anesthetized as described above. After checking for proper anesthesia, the animal was turned on its back and restrained using surgical tape. Hair on the thigh area was removed using a hair removal lotion (Nair), then disinfected using an iodine solution. An incision was made on the right thigh area above the femoral artery. The femoral artery was then isolated and cleared of connective tissue. Blood flow is temporarily stopped using a small artery clamp, and an incision was made on a branch between the rectus femoris and vastus medialis muscles [14]. A fixed core wire guide coated with heparin was inserted into the artery and moved up. The artery clamp was then removed and the wire moved up toward the iliac artery. The wire was then allowed to stay for 60 s, then pulled back towards the incision point. This insertion and pulling back were repeated for a total of three times. At the end of the third time, the artery was once again clamped and the wire guide pulled out of the artery. The incision site on the artery was then ligated using surgical silk (5–0 Vicryl). The skin was then closed and sutured together, then sealed with surgical glue. A similar procedure was performed on the left leg, with the exception that the wire was not inserted into the artery.

The femoral artery from the left leg was used as control.

Tissue processing

After euthanasia via CO_2, animals were injected with 0.1 mL of heparin and flushed of blood using ice-cold PBS via the left ventricle. The animals were then perfused with 4% paraformaldehyde. Both femoral arteries were removed from the body and fixed overnight in 4% paraformaldehyde, prior to being embedded in paraffin and sectioned at 5 μm. For histological analysis on acute samples, harvested femoral arteries were directly embedded in OCT and subjected to slicing at 5 μm by cryostat sectioning

H&E staining and immunostaining

H&E staining was performed by the Translational Pathology Core Laboratory Core Facility at UCLA using standard protocols, following sectioning. Further, PCNA immunostaining was performed using VECTASTATIN ABC Kit (Vectr Laboratories, Burlingame, CA). To observe the acute response after injury, CD34 and Isolectin GS-IB4 antibodies were used to detect homing EPCs and mature endothelial cells, respectively, on 1-h post-injury tissues. In

addition, EPCs isolated from GFP transgenic mice were also subjected to netrin-1 preconditioning prior to injection, and the homing to the wound site was determined by co-staining with anti-GFP antibody and anti-Isolectin GS-IB4 antibody.

Quantification of neointimal formation

Using Image J software, the areas of the intimal and medial layers were measured manually. Ratio of intima to media was used for quantitative assessment of neointimal formation.

Nitric oxide radical detection by electron spin resonance

Bioavailable $NO^{\bullet}$ radical in femoral arteries and EPCs was detected using electron spin resonance (ESR) as previously described [8, 15] with modifications for the current experimental setup. For in vivo experiments, femoral arteries from four animals were combined for one experimental data point. $NO^{\bullet}$ production was measured from non-injured with or without netrin-1 injection (100 ng/ml of recombinant netrin-1 injected via the tail vein immediately after femoral injury surgery) and injured with or without netrin-1 injection. For in vitro experiments using EPCs, two 10-cm culture dishes were combined for each

group. The tissues or cells were incubated with freshly prepared $NO^{\bullet}$-specific spin trap Fe^{2+} $(DETC)_2$ (0.5 mmol/L) colloid in modified Krebs/HEPES buffer with 50 μM (femoral artery) or 10 μM (EPCs) of calcium ionophore A23187 (Sigma, St Louis, MO) at 37 °C for 3 (femoral artery) or 1.5 (EPCs) hours. After incubation, tissues or cells were snap frozen in liquid nitrogen, then loaded into a finger Dewar for ESR measurement. The instrument settings were as follows: bio-field, 3410.00 G; field sweep, 100 G; microwave frequency, 9.79 GHz; microwave power, 13.26 mW (6 dB); modulation amplitude, 9.82 G; resolution, 512 points; and receiver gain 356.

Co-culture of VSMCs and endothelial cells

Rat aortic smooth muscle cells (Lonza, Switzerland) were cultured in DMEM/F12 medium (Invitrogen) containing 15 ng/ml of amphotericin and 10% fetal bovine serum to confluence and starved in serum-free medium overnight before experiments. Bovine aortic endothelial cells (BAECs) (CellSystems, Kirkland, WA) were cultured as described previously [16]. Confluent VSMCs and endothelial cells were mixed together with equal cell number for migration assay.

VSMC migration assay

Transwell migration chamber (8-μm pore size, Corning) in 24-well plates was used for migration assay. Confluent cells were starved with serum-free medium overnight prior to plating to the upper chamber. Co-culture of VSMCs and endothelial cells were seeded at a density of 2–2.5 × 10^5 cells/well on the top of the insert in 350 μl of serum-free culture medium, while 1 ml of 5% FBS medium containing pharmacological inhibitors [U0126 (50 μM), SB202190 (10 μM), SP600125 (10 μM), Rp-8-Br-PET-cGMPs (10 μM) and PTIO (60 μM)] was placed in the lower chamber. After 30 min of incubation, netrin-1 (100 ng/ml) was added into the medium in the lower chamber. The plates were then incubated for 24 h, followed by treatment of the cells with a dissociation buffer (Trevigen, Gaithersburg, MD) containing calcein AM (Calbiochem, Germany) to detect and quantify the fluorescence of migrated cells. The fluorescence were measured in a Bio-Tek fluorescence plate reader set at an excitation wavelength of ~485 nm and an emission wavelength of ~520 nm with triplicate readings.

Wound healing assay on separately co-cultured VSMC with EC

Instead of mixing BAECs and VSMCs together, we also cultured 2 types of cells separately toinvestigate proliferation/migration activity only from VSMCs. The bottom surface of 24 mm polyester membrane transwell inserts with 0.4 μm pore (Corning) was coated with 0.05% gelatin, and BAECs were seeded on top of the bottom surface by placing the inserts up-side-down. After culturing BAECs on the transwell membrane for overnight, the insert was placed back to a normal 6 well-plate containing culture medium, and continued to culture until confluent. Whereas VSMCs were cultured on 6 well-plate till more than 80 % confluent. When the both cells are ready, a scratch was made on VSMCs using 1 mL pipet tip. Culture medium for VSMCs were replaced to low glucose (1 g/L) DMEM since BAECs could be damaged by the high dose of glucose. Then the scratch on VSMCs were imaged using Nikon Eclipse Ti confocal microscope. After imaging the initial state of wound, VSMCs were pre-treated with or without SB202190 (10 μM), Rp-8-Br-PET-cGMPs (10 μM) and PTIO (60 μM), while BAECs were treated with or without netrin-1 (100 ng/mL). Thirty minutes later, the transwell inserts with BAECs were replaced on the 6 well-plate contains VSMCs and incubated at 37 °C with a 5% CO_2 incubator for 24 hrs. Another image of the wound was taken after 24 hrs and the area of wound healing was calculated using ImageJ. The illustrated figure of this experiment is shown in Figure 2-2.

Western blot analysis on co-cultured cells

Co-cultured cells were starved overnight in 0.5% FBS containing DMEM, incubated with or without Rp-8-Br-PETcGMPs (10 μM, 30 min), and then exposed to netrin-1 (100 ng/ml) for 24 h. After treatment, cells were harvested and lysed, and subjected to detection of total p38 MAPK and phospho-p38 MAPK protein levels by Western blotting per standard protocols using 12% SDS/PAGE and nitrocellulose membranes. Antibody dilutions used were as follows: p38 MAPK (1:1000) and phospho-p38 MAPK (1:1000); alpha-Tublin (1:1000) was used as internal control. Goat anti-rabbit IgG-HRP conjugate (1:3000) was used as the secondary antibody. The blotting results were quantified using ImageJ, normalized to alpha-Tublin, and statistically analyzed from four independent experiments.

Figure 2-2.

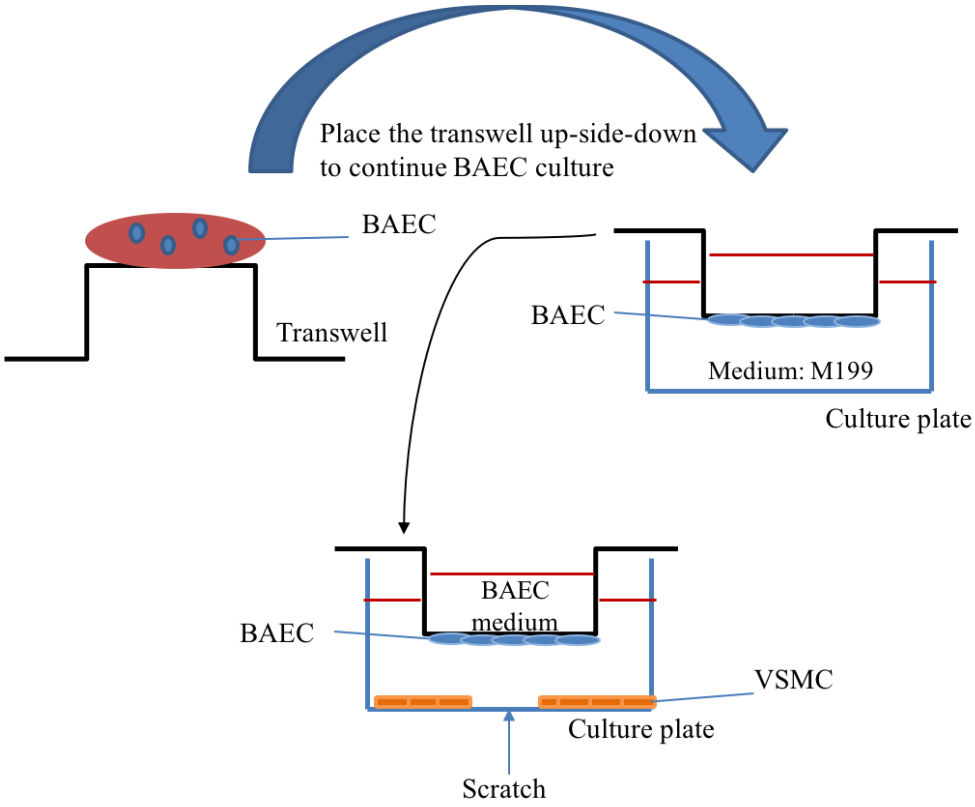


Figure 2-2. Scratch assay in separate co-culture system of BAEC and VSMC. BAECs were cultured on the bottom surface of transwell by placing it up-side-down. The tranwell was replaced on 6 well-plate for continuing culture of BAECs. VSMCs were cultured on the bottom of a different 6 well-plate. The transwell with BAECs was transferred on the culture plate with VSMCs before scratch assay. A scratch was made on VSMC with 1 mL pipet tip.

EPC isolation and culture

Mononuclear cells were collected from bone marrow of femur and tibia of 6–8-week-old C57BL6 mice (WT) or $DCC^{+/-}$ mice using density centrifugation and cultured in EGM-2 medium (Lonza). The non-adherent cells were removed after 48 h, and then colony forming cells became confluent on the culture plate after 7–10 days. EPCs were characterized by immunocytochemical staining with CD34, CD133, Flk-1, and vWF antibodies. The receptor expression response to netrin-1 was investigated on cultured EPCs.Cells were harvested 30 min after treating with or without netrin-1 (100 ng/mL) and extracted proteins per standard western blot protocol. Anti-DCC antibody (Santa Cruz Biotechnology/ 1:100 dilution) and anti-UNC5B antibody (Enzo Life Sciences/ 1:500 dilution) were used to detect their expression in EPCs.

EPC injection

Primarily, cultured EPCs isolated from WT or GFP transgenic mice were preconditioned with netrin-1 (100 ng/ml, 1 h) and 500 of the unconditioned and conditioned cells were injected through the tail vein of the animal immediately after femoral injury surgery. One hour or 4

weeks later, femoral arteries were harvested and processed for histological analyses of immunofluorescent or H&E staining, and re-endothelialization or the intimal growth was quantified.

EPC proliferation assay

EPCs from $DCC^{+/+}$ and $DCC^{+/-}$ animals were seeded at an initial density of 1.0×10^5 cells/well in 12-well plates and incubated at 37 °C with a 5% CO_2 incubator. After overnight incubation to allow cells to attach to the bottom of the plates, cells were starved with 5% FBS contained endothelial growth basal medium-2 (EBM-2) for 12 h. Then, cells were additionally incubated with or without netrin-1 (100 ng/ml in 5% FBS contained EBM-2 medium) for 24 h, following a pretreatment with or without PTIO (60 μM, 30 min). At the end of the incubation, cells in each well were resuspended following trypsinization and counted using a hemocytometer. Individual experiment was prepared in triplicate and was repeated three times.

EPC apoptosis assay—caspase 3 assay

EPCs from $DCC^{+/+}$ and $DCC^{+/-}$ animals were seeded at approximately 1.0×10^5 cells/well in 24-well plates and cultured for 3 to 4 days till the cells become more than 90% confluent. Cells were then starved with 5% FBS contained EBM-2 medium. After overnight starvation, medium was discarded and replaced with PBS with or without PTIO (60 μM, 30 min) and/or netrin-1 (100 ng/ml, 30 min), and then exposed to hydrogen peroxide (H_2O_2, 500 μM) for 30 min. Caspase 3 activity was assessed with a fluorimetric caspase 3 Assay Kit (Sigma), following the manufacturer's instruction. Briefly, cells were suspended into tubes with lysis buffer. Assay buffer and caspase 3 substrate Ac-DEVD-AMC were added and the sample mixtures were transferred to a black 96-well plate along with substrate blank and caspase 3 positive control. The fluorescent signal from AMC, which is the hydrolysed substrate by caspase 3, was detected at excitation of 360 nm and emission of 460 nm every 10 min for 1 h.

EPC apoptosis assay—TUNEL assay

APO-BrdU TUNEL assay (Invitrogen) was performed using the identically treated EPCs subjected to caspase 3 assay. The kit detects the DNA fragmentation of apoptotic cells by reacting terminal deoxynucleotidyl transferase (TdT) and thedeoxythymidine analog (BrdUTP) to label the break sites. The incorporated BrdU at DNA break sites was detected

by an Alexa Fluor 488 dye-labeled anti-BrdU antibody. Therefore, the Alexa Fluor 488 dye along with propidium iodide (detects the total cellular DNA content) was imaged with the Nikon Eclipse Ti confocal microscope.

eNOS phosphorylation in EPCs

Alongside, NO radical detection by ESR, eNOS phosphrylation at serine 1179 in mouse EPCs was examined using Western blot analysis. Antibody dilutions used were as follows: eNOS (1:500) and phospho-eNOS (Ser 1179) (1:500). Actin (1:10,000) was used as an internal control. Goat anti-rabbit IgG-HRP conjugate (1:3000) was used as the secondary antibody.

Statistics

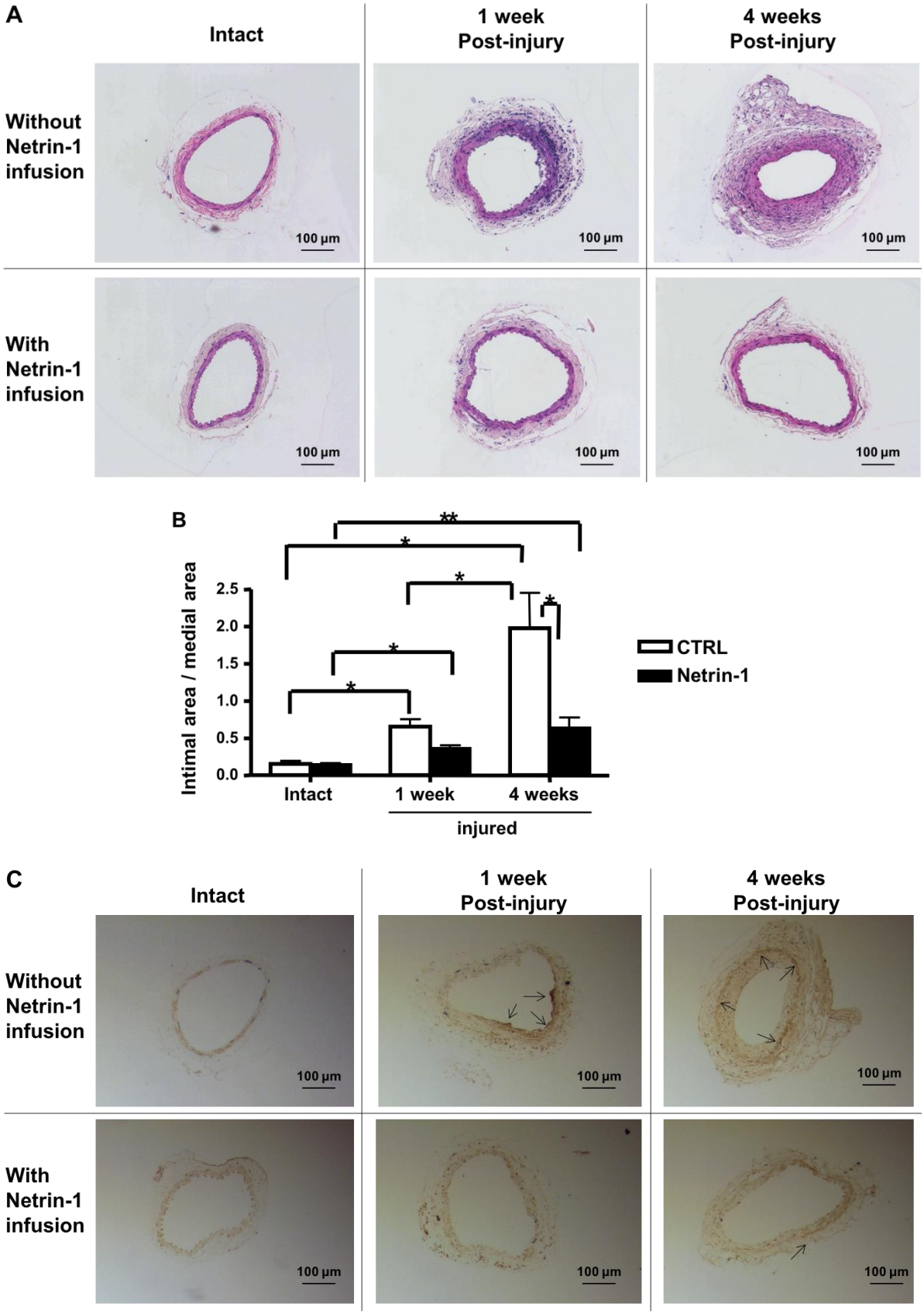
All statistics were performed using Prism software. Comparisons between two groups were performed using t tests, with a significance level of 0.05. Comparisons among multiple groups were performed using ANOVA, with Newman-Keuls as the post hoc test and a significance level of 0.05.

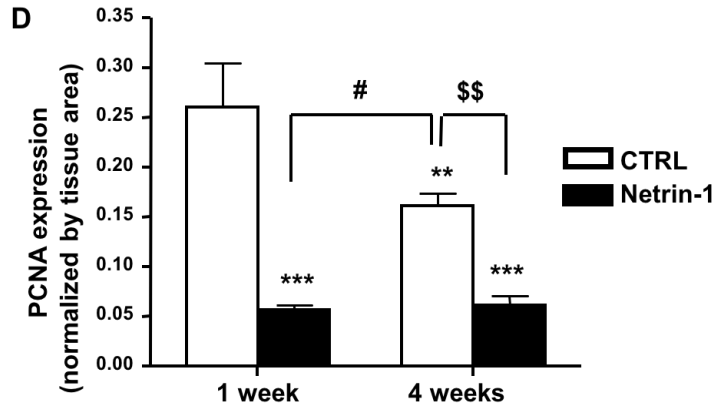
2-3. Results

Netrin-1 infusion markedly attenuated neointimal formation post endothelial injury

To test whether netrin-1 induces protection against neointimal growth, femoral wire injury was performed on animals that were infused with netrin-1 or vehicle. Intima to media ratio (averaged at 0.15 ± 0.05 for intact vessels) was 1.98 ± 0.47 at 4 weeks after surgery in vehicle-treated animals, while netrin-1 infusion markedly decreased this ratio to 0.64 ± 0.14 (Figure. 2-3A, B). Netrin-1 was also effective in reducing neointimal growth even at week 1. The representative H&E images of the vessels which are shown in Figure. 2-3A, C, D demonstrate that VSMC proliferation in situ, labeled by PCNA, was significantly increased in wire injured femoral arteries. This was, however, largely prevented by netrin-1 infusion.

Figure 2-3.


A
Intact
1 week
Post-injury
4 weeks
Post-injury
Without
Netrin-1
infusion
With
Netrin-1
infusion
100 μm
B
Intimal area / medial area
2.5
2.0
1.5
1.0
0.5
0.0
Intact
1 week
4 weeks
injured
CTRL
Netrin-1
C
Intact
1 week
Post-injury
4 weeks
Post-injury
Without
Netrin-1
infusion
With
Netrin-1
infusion
100 μm



Figrue 2-3. Netrin-1 infusion significantly inhibits neointimal growth after femoral artery wire injury. Femoral Femoral artery was injured by the passing of a guide wire, and netrin-1 was infused via an osmotic minipump. Femoral arteries were harvested after 1 or 4 weeks, fixed in paraformaldehyde, and sectioned and stained for H&E and PCNA antibody. **A)** Representative H&E images. **B)** Grouped data of intima to media ratio. Data show that at 1 and 4 weeks, netrin-1 infused animals had significantly smaller intima to media ratio in the wire damaged artery, indicating inhibition of neointimal growth. n=4-8. *p<0.05 ,**p<0.01 **C)** Representative images of immunohistochemical staining. PCNA positive cells are stained with dark brown in color. **D)** Grouped quantitative data of PCNA-positive cells to whole tissue ratio. Netrin-1 infused animals had significantly less PCNA both at 1 and 4 weeks post-injury. n=4-7. **p<0.01, ***p<0.001 v.s. CTRL in 1 week, $^{\#}$p<0.05, $^{\$\$}$p<0.01

Netrin-1's protective effect against neointimal formation is dependent on DCC

To test whether netrin-1 receptor DCC is involved in the protective effect to attenuate neointimal formation, similar experiments described above were performed in $DCC^{+/+}$ and $DCC^{+/-}$ animals. The results, shown in Figure. 2-4A for representative H&E images and Figure. 2-4B for quantitative data, indicate that netrin-1's inhibition on neointimal growth was abolished in $DCC^{+/-}$ animals. This confirms that DCC is the receptor through which netrin-1 exerts its vascular protective effects against wire-induced endothelial injury.

Figure 2-4.

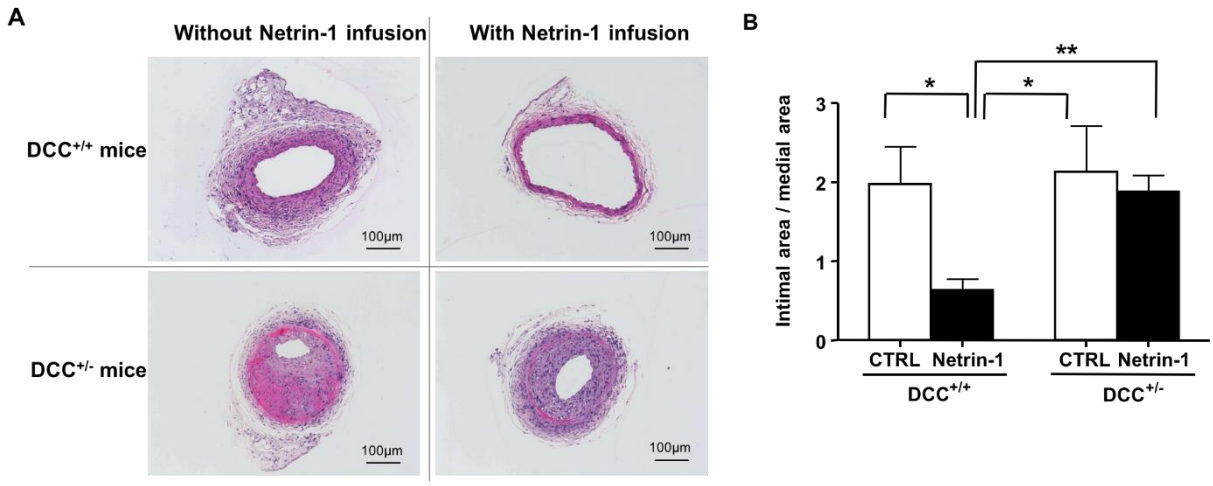


Figure 2-4. Netrin-1's protective effect against neointimal growth is mediated by the receptor DCC. Netrin-1 was infused via an osmotic minipump into $DCC^{+/+}$ and $DCC^{+/-}$ mice that received a femoral wire injury. The femoral arteries were harvested 4 weeks later, fixed in paraformaldehyde, sectioned, and stained for H&E. **A)** Representative H&E images;**B)** Grouped data for intima to media ratio from $DCC^{+/+}$ and $DCC^{+/-}$ animals, indicating that netrin-1 induced attenuation of neointimal growth was absent in $DCC^{+/-}$ mice. n=4-9 each, *p<0.05, **p<0.01

Netrin-1 significantly induces NO• production in femoral arteries

In previous studies in our laboratory, we found that netrin-1 can increase NO production both in cells [8] and tissue [7, 9–13]. Since NO• is known to attenuate neointimal formation [16, 17], we hypothesized that NO•mediates netrin-1's inhibitory effect on neointimal formation in the wire injury model. Here, we measured NO•production using ESR from femoral arteries 1 h after injury, in the presence or absence of netrin-1 intravenous injection (immediately after surgery, 100 ng/ mL blood). As shown in Figure. 2-5, we observed a 38.5% reduction in NO•bioavailability in the injured femoral arteries, indicating loss of endothelium and eNOS. Netrin-1 treatment elevated NO•production in both injured and non-injured vessels, implicating potent activation of eNOS in both intact and partially remained endothelium.

Figure 2-5.

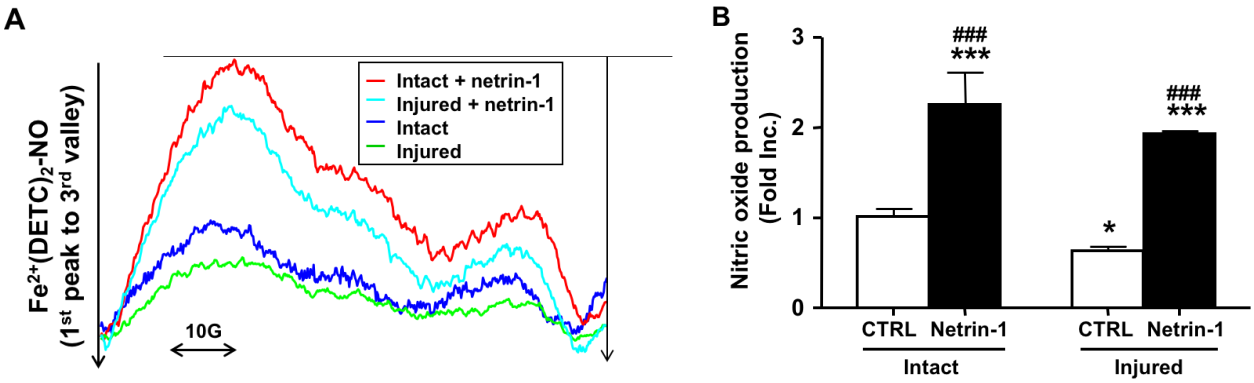


Figure 2-5. Netrin-1 infusion increases NO production. Netrin-1 was injected via tail vein immediately after injury. The harvested 1 hr post-injured femoral arteries were incubated in the spin trap solution for 3 hours before electron spin resonance (ESR) determination of NO production. **A)** Representative ESR spectra; **B)** Grouped data for NO production. n=4-10 each, *p<0.05, ***p<0.001, vs. CTRL, intact, $^{###}$p<0.001, vs. CTRL, injured

Netrin-1 inhibits VSMC migration via an NO•-dependent mechanism

Migration of VSMC is one of the major events that leads to abnormal intimal growth after endothelial damage. In a previous study, we demonstrated increased NO•production in netrin-1 endothelial cell [8], which has been shown to be a potent inhibitor of VSMC migration. Hence, we examined whether netrin-1's protective effect on neointimal growth is related to inhibition of VSMC migration by NO•. We co-cultured VSMCs with endothelial cells, treated the cells with vehicle, netrin-1, or netrin-1 in combination with PTIO, an NO•scavenger, and then the migrated cells were dissociated and quantified by detecting fluorescence from calcein AM. The results, shown in Figure. 2-6A, demonstrate that netrin-1 significantly and reproducibly inhibited cell migration from these co-cultures. Further, co-treatment with PTIO largely abolished this inhibition, suggesting that netrin-1's effect on VSMC migration is mediated via NO•. We further examined the downstream signaling pathways that lead to the inhibition of migration in VSMCs by pretreating VSMCs with inhibitors against cGMP, and those of major mitogenic pathways. The results in Figure. 2-6A show that while inhibitors against ERK1/2 and JNK had no effect on netrin-1's inhibition of VSMCmigration, blockade of cGMP and p38 MAPK signaling abolished netrin-1's effect.

Although the mixed co-culture presented above is simulating the actual biology of vascular where the endothelial cells and VSMCs are attached to each other, it is also important to focus on the kinetics of VSMCs influenced by the secondary effect generated from endothelial cells. In this context, our purpose was to deliver $NO^{\bullet}$ produced from endothelial cells by netrin-1 treatment and diffuse into VSMCs. Therefore, transwell insert was used in this experiment (experimental model: Figure 2-2). VSMCs were modestly but significantly inhibited the wound healing by co-culturing with netrin-1 treated BAECs, but this effect was diminished by pre-treating VSMCs with PTIO, cGMP antagonist, and p38 MAPK inhibitor (Figure 2-6C, D). This result is reproducing the mixed co-culture experiments, confirming the involvement of p38 MAPK and cGMP in inhibition of VSMC migration by $NO^{\bullet}$ production in endothelial cells treated with netrin-1.

We next examined the relationship between cGMP and p38 MAPK. Using Western blotting, we examined the phosphorylation levels of p38 MAPK in the EC-VSMC co-cultures treated with netrin1 in the absence or presence of the cGMP antagonist. The results in Figure. 2-6b demonstrate that cGMP antagonist attenuated p38 MAPK activation, implicating a $NO^{\bullet}$/ cGMP/p38 MAPK pathway in mediating netrin-1- induced inhibition of VSMC migration.

Figure 2-6.

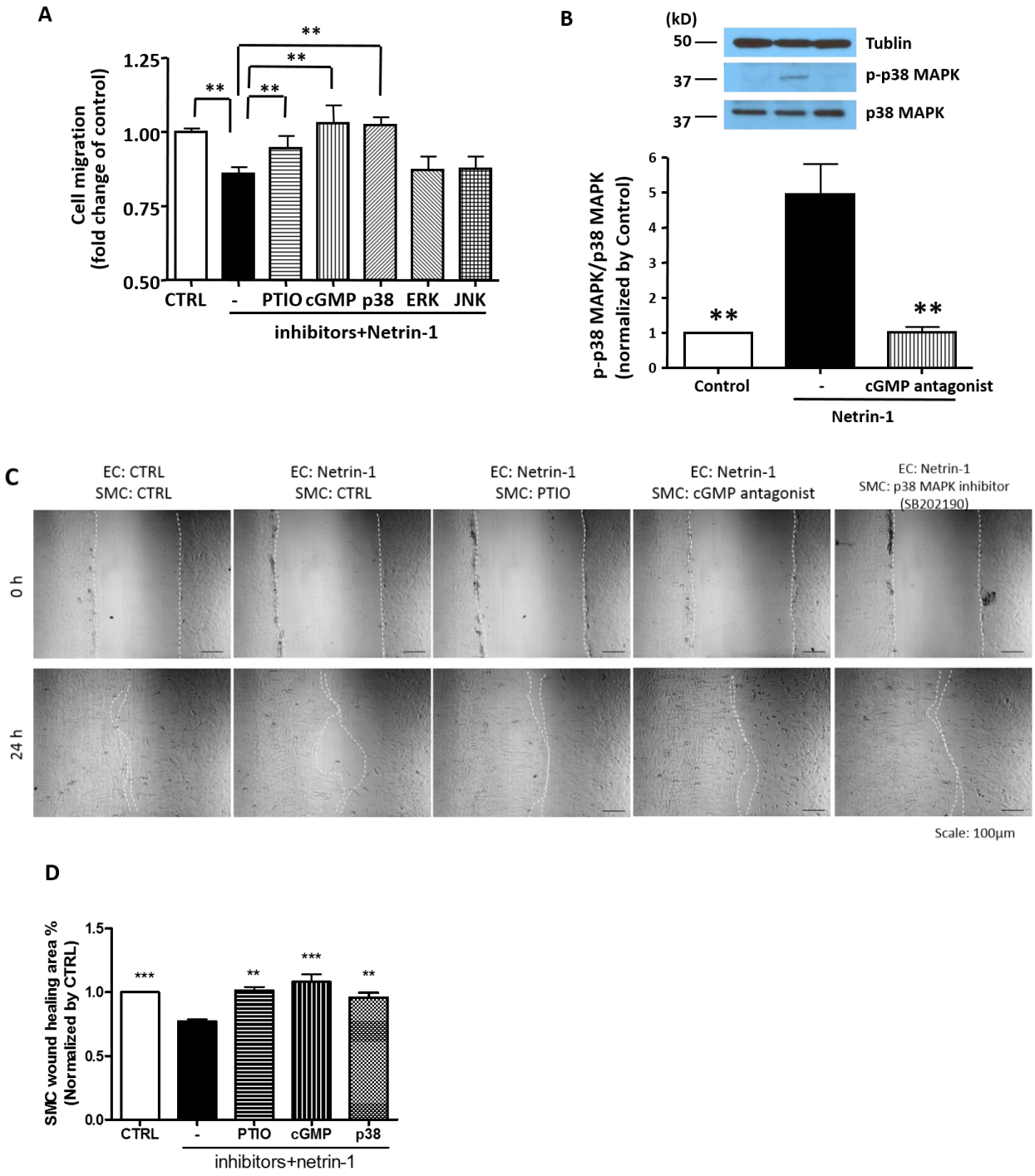

A
Cell migration (fold change of control)
1.25
1.00
0.75
0.50
**
**
**
**
CTRL
-
PTIO
cGMP
p38
ERK
JNK
inhibitors+Netrin-1
B
(kD)
50
37
37
Tublin
p-p38 MAPK
p38 MAPK
p-p38 MAPK/p38 MAPK (normalized by Control)
6
5
4
3
2
1
0
**
**
Control
-
cGMP antagonist
Netrin-1
C
EC: CTRL
SMC: CTRL
EC: Netrin-1
SMC: CTRL
EC: Netrin-1
SMC: PTIO
EC: Netrin-1
SMC: cGMP antagonist
EC: Netrin-1
SMC: p38 MAPK inhibitor
(SB202190)
0 h
24 h
Scale: 100μm
D
SMC wound healing area %
(Normalized by CTRL)
1.5
1.0
0.5
0.0
***
**
***
**
CTRL
-
PTIO
cGMP
p38
inhibitors+netrin-1

Figure 2-6. Netrin-1 inhibits migration of vascular smooth muscle cells.

Vascular smooth muscle cells (VSMCs) and endothelial cells (ECs) were co-cultured in equal cell numbers. Cell migration was measured using transwell assay. **A)** VSMC migration from VSMC/EC co-cultures treated with netrin-1 in the absence or presence of pharmacological inhibitors (PTIO (60uM) as NO scavengers, and inhibitors for p38 MAPK (10uM), cGMP (10uM), ERK (50uM), JNK (10uM)) **$p<0.05$, n=3-4. Data indicate NO, cGMP and p38 MAPK-dependent attenuation of VSMC migration by netrin-1. **B)** Western blot analysis for p-p38 MAPK/p38 MAPK in netrin-1 treated co-cultures in the presence or absence of cGMP antagonist Rp-8-Br-PET-cGMPs, indicating cGMP lies upstream of p38 MAPK. n=3, *$p<0.01$, **$p<0.05$ vs. netrin-1 **C)** VSMC wound healing assay was performed with a separate co-culture system showed in Figure 2-2. **D)** The quantitative result of C) from n=6. ** $p<0.01$, *** $p<0.001$, vs. netrin-1 treatment alone.

Netrin-1 infusion accelerates timely homing of EPCs to the wound site

Timely re-endothelialization after endothelium damage is critical in the prevention of vascular remodeling. Hence, there must be a more acute modification by netrin-1 that helps re-endothelialization, so that the inhibition of VSMC migration can be triggered. Figure 2-7 shows increased expression and colocalization of CD34 (a EPC marker) with Isolectin (an EC marker) in netrin-1 infused injured femoral arteries 1-h post-injury and EPC injection (Figure. 2-7D'), whereas neither of those were present at the endothelial layer of the injured femoral arteries without netrin-1 infusion (Figure. 2-7C'). Therefore, the results indicate that netrin-1 accelerated timely homing and re-endothelialization of EPCs to the wound site. To further examine homing of EPCs from the injected population, EPCs isolated from GFP transgenic mice (GFP-EPCs) were traced after injecting into WT animals by imaging GFP. As shown in Figure. 2-7E–G, homing of GFP-EPCs to the injured femoral arteries was accelerated by netrin-1 preconditioning.

Figure 2-7.

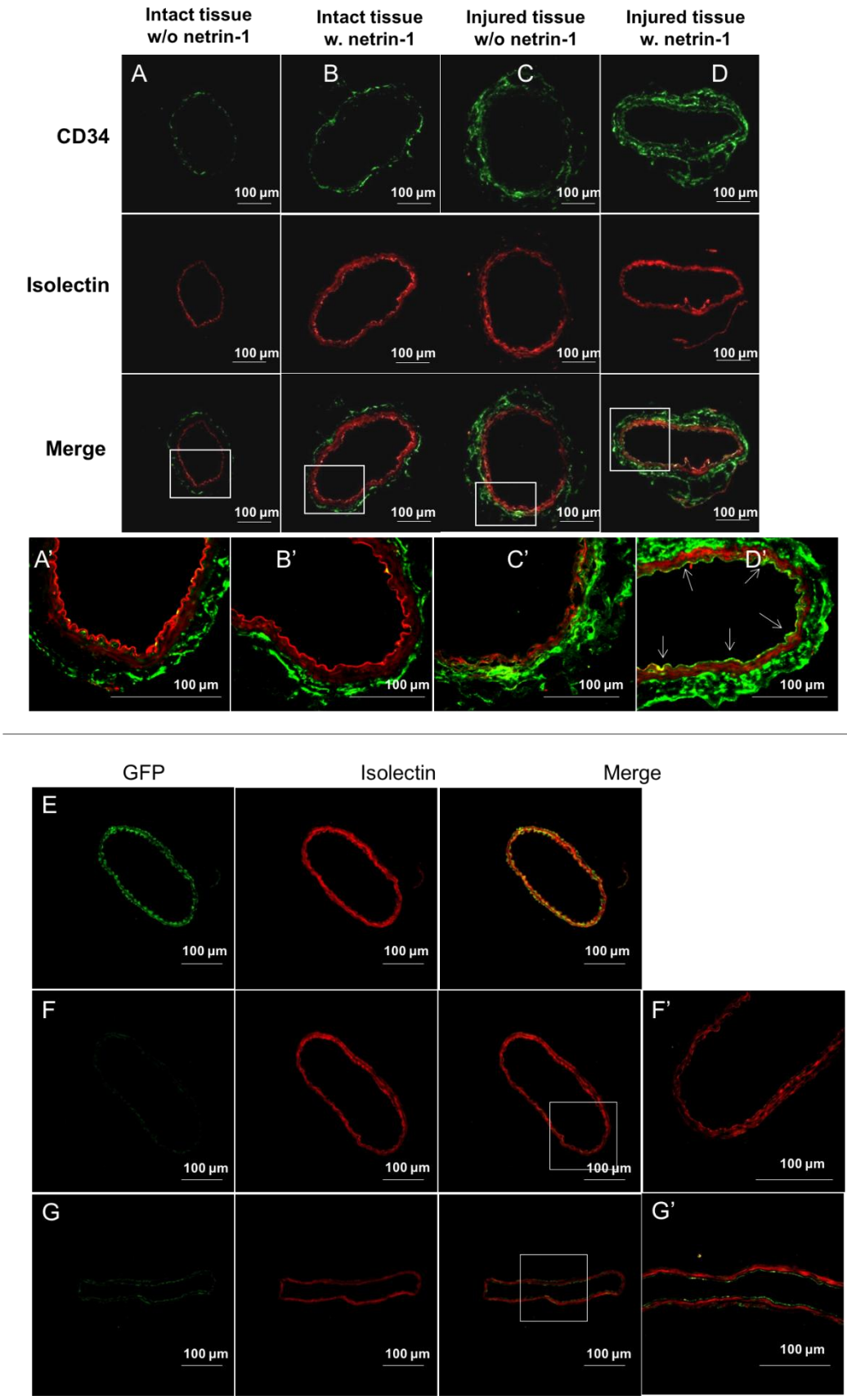

Intact tissue w/o netrin-1
Intact tissue w. netrin-1
Injured tissue w/o netrin-1
Injured tissue w. netrin-1
CD34
Isolectin
Merge
A
B
C
D
A'
B'
C'
D'
100 µm
GFP
Isolectin
Merge
E
F
F'
G
G'

Figure 2-7. Netrin-1 accelerated EPC homing after endothelial injury. Femoral arteries were harvested at 1 hr post-injury, with or without netrin-1 pre-infusion. EPC homing was evaluated by co-staining with anti-CD34 and Isolectin antibodies. Shown here are the representative immunohistochemical images from each group. **A)** Intact tissue without netrin-1 infusion. **B)** Intact tissue with netrin-1 infusion. **C)** Injured tissue without netrin-1 infusion. **D)** Injured tissue with netrin-1 infusion. Data indicate accelerated EPC homing, implicated by co-localization of CD34 and Isolectin at the endothelial layer of netrin-1 infused injured femorals, but not on the injured femorals without netrin-1 infusion. Moreover, Femoral arteries were harvested at 1 hr post-injury, with or without netrin-1 pre-condition on EPC isolated from GFP transgenic mice. EPC homing was evaluated by co-staining with anti-GFP and Isolectin antibodies. **E)** Showing an intact femoral artery from GFP transgenic animal as a comparison. **F)** Injured tissue received GFP-EPC without netrin-1 pre-condition. **G)** Injured tissue received GFP-EPC with netrin-1 pre-condition. Data indicate accelerated EPC homing by netrin-1 pre-condition, implicated by co-localization of GFP and Isolectin at the endothelium of injured femorals, but not on injured femorals without netrin-1 pre-conditioning. **A') – D'), F') and G')** Zoomed-in pictures of selected areas. The pictures were inverted due to the use of inverted confocal microscope with 40x object lens.

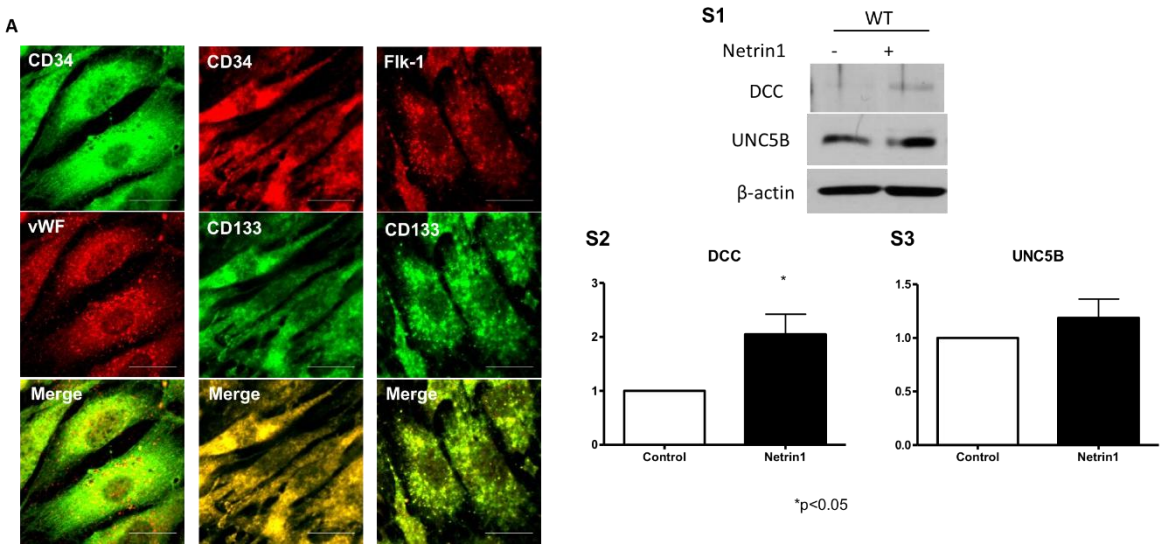
Netrin-1 preconditioned EPCs attenuate wire injury-induced neointimal formation

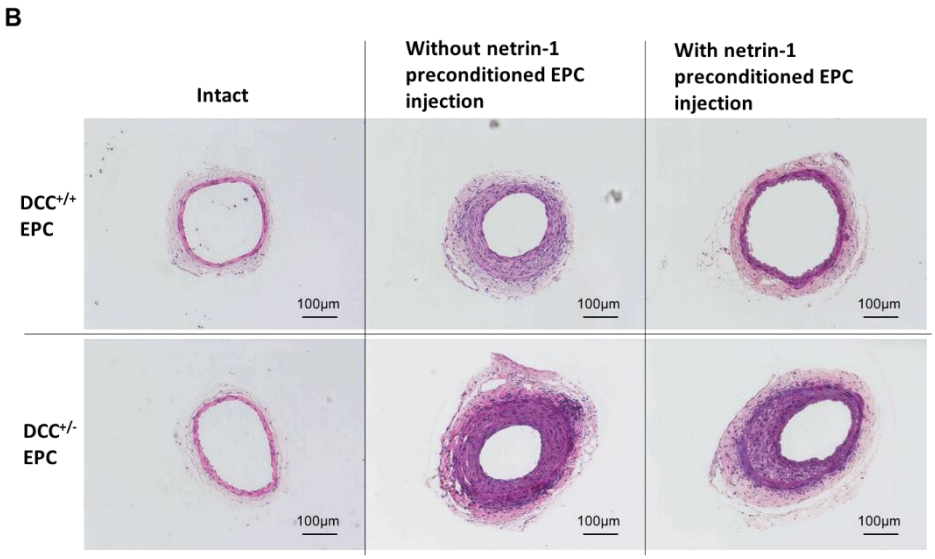
EPCs play an important role in the re-endothelialization process [19]. EPCs derived from bone marrow mobilize to peripheral circulation and differentiate into mature ECs once they attach to the wound site. Therefore, timely recruitment of EPCs to the damaged vessel is essential to the prevention of neointimal growth [18]. Hence, another mechanism by which netrin-1 may exert its protective effect is via functionalaugmentations of EPCs. Here, we examined whether netrin-1 preconditioned EPCs can confer vascular protection. EPCs isolated from either $DCC^{+/+}$ or $DCC^{+/-}$ animals were injected into WT mice via tail vein immediately after wire injury. The femoral arteries were harvested after 4 weeks to assess intima to media ratio. Figure 2-8A shows the characterization of bone marrow-derived EPCs, which express CD34, CD133, Flk-1, and vWF. As supporting information to examine whether EPCs respond to netrin-1, expressions of two representative receptors of netrin-1, DCC and UNC5B, was detected. Figure. 2-8Sshows EPCs does respond to netrin-1 and only DCC expression level was significantly increased by netrin-1 treatment, while UNC5B protein abundance ramined unchanged.

Figure 2-8B shows representative H&E images of the femoral arteries, while Figure. 2-8C shows summarized data. The results indicate that netrin-1 preconditioned EPCs

largely attenuated neointimal formation, which was absent when EPCs isolated from the $DCC^{+/-}$ animals were used, suggesting that netrin-1 can directly impinge on EPCs to exert the beneficial effect, and that the EPCs also require DCC receptor to respond to netrin-1 preconditioning. These results implicate a role of EPCs in the repair mechanism and suggest that netrin-1 preconditioned EPCs may serve as a potential therapeutic application.

Figure 2-8.


A
CD34
CD34
Flk-1
vWF
CD133
CD133
Merge
Merge
Merge
S1
WT
Netrin1
-
+
DCC
UNC5B
β-actin
S2
DCC
S3
UNC5B
Control
Netrin1
Control
Netrin1
*p<0.05


B
Intact
Without netrin-1 preconditioned EPC injection
With netrin-1 preconditioned EPC injection
DCC+/+ EPC
DCC+/- EPC
100μm

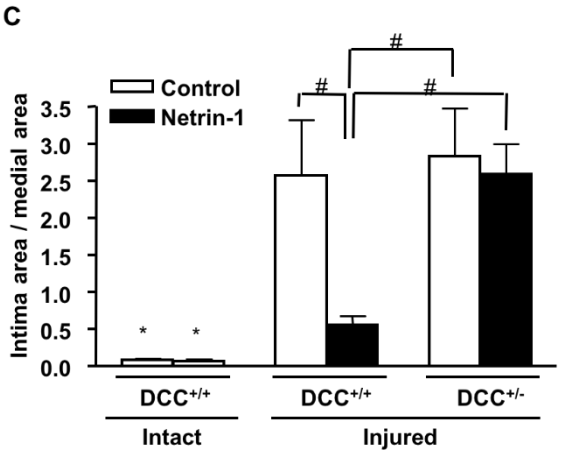

C
Intima area / medial area
Control
Netrin-1
DCC+/+
DCC+/+
DCC+/-
Intact
Injured

Figure 2-8. Netrin-1 preconditioned EPCs significantly reduces neointimal growth in a DCC-dependent fashion. **A)** Characterization of EPCs isolated and differentiated from mouse bone marrow (Scale: 20 μm). These EPCs express CD34, CD133, vWF, and Flk-1, which colocalize well with each other. Particularly, vWF is localized to Weibel-Palade bodies. **S1-3)** DCC and UNC5B receptor response to netrin-1 was confirmed in cultured EPCs using western blot.Netrin-1 treatment at the concentration of 100 ng/mL significantly increased the expression of DCC, but no change in UNC5B. **B)** EPCs isolated from $DCC^{+/+}$ or $DCC^{+/-}$ mice were preconditioned with netrin-1 (100 ng/ml, 1 hr) prior to being tail vein injected into wild type animals immediately after femoral wire injury. The femoral arteries were harvested after 4 weeks, fixed, sectioned, and stained for H&E. Showing the representative H&E images; **C)** Grouped data of intima to media ratio, indicating that netrin-1 treated EPC markedly attenuated neointimal growth, while this protective effect was abolished with EPCs harvested from $DCC^{+/-}$ animals.

Netrin-1 significantly improves EPC proliferation

As endothelial cells are potent inhibitors of VSMC migration, timely regeneration of the endothelial layer is critical in preventing neointimal growth after vascular injury. Hence, the proliferation of EPCs is one of the critical factors that are involved in re-endothelialization and vascular repair. Here, we examined whether netrin-1 can improve proliferation of EPCs. The results, shown in Figure2-9A, B, indicate that netrin-1 (100 ng/ml, 24 h) significantly increased $DCC^{+/+}$-EPC proliferation by 42%, which was also mediated by $NO^{\bullet}$ as $NO^{\bullet}$scavenger PTIO attenuated this response. We have previously shown that netrin-1 can promote endothelial cell proliferation and migration through its receptor DCC via an ERK1/2- $NO^{\bullet}$pathway [8]. In this study, we examined proliferation of WT EPCs and those isolated from $DCC^{+/-}$ animals. The EPCs isolated from $DCC^{+/-}$ mice did not show improvement in proliferation in response to netrin-1 treatment (Fig. 2-9B). Therefore, the beneficial response to netrin-1 in EPC proliferation requires the presence of DCC.

Netrin-1 significantly improves EPC resistance to oxidative stress-induced apoptosis

It has been shown that EPCs are sensitive to oxidative stress-induced apoptosis [19], and its number is oftenreduced in patients with coronary artery disease [20, 21]. Therefore, it is important to augment the resistance to oxidative stress of EPCs to ensure their viability to promote vascular repair. Hydrogen peroxide (H_2O_2)-induced apoptosis in EPCs was measured using a caspase 3 assay. It turned out H_2O_2 increased caspase 3 activity by 43%, which was abolished by netrin-1 treatment (Fig. 2-9C). TUNEL assay also showed a significant decrease of H_2O_2-induced apoptosis in netrin-1-treated EPCs (Fig. 2-9D, representative images in Fig. 2-9E). For both assays, the protective effect of netrin-1 was absent with PTIO scavenging of $NO^{\bullet}$. These data clearly demonstrate that netrin-1 protects EPCs against apoptosis via $NO^{\bullet}$.

Figure 2-9.

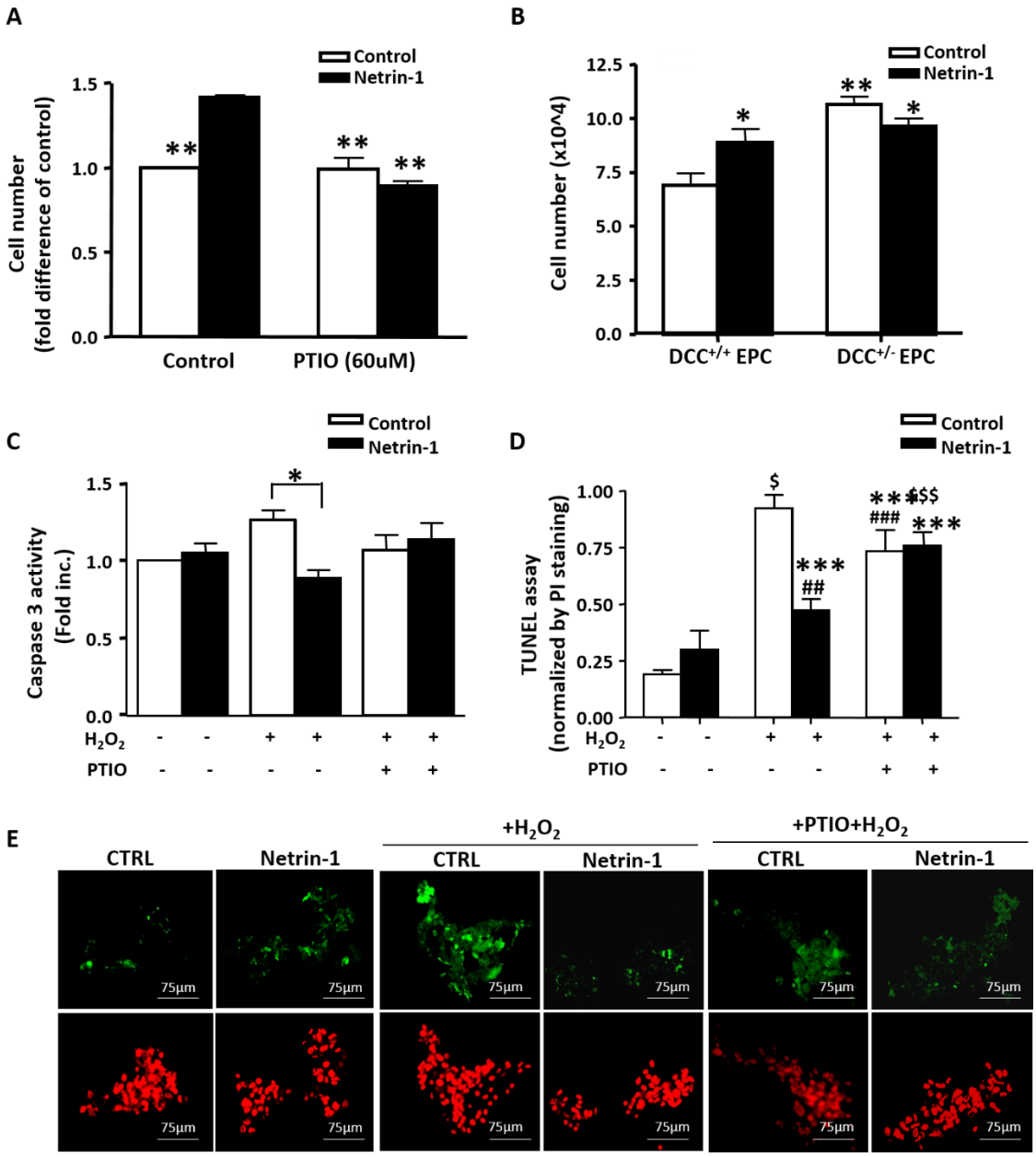


Figure 2-9. Netrin-1 augments EPC function: increased proliferative activity and resistance to oxidative stress induced apoptosis. Netrin-1 was used to pretreat the cells for 30 min prior to H_2O_2 stimulation. Cells were cultured with PTIO (60 µM) for 30 min before netrin-1 treatment in order to scavenge NO generated by netrin-1 treatment. **A)**

Proliferation of wild type EPCs was measured by cell number using hemocytometer. Netrin-1 increased the proliferative activity of EPCs, while having no effect on EPCs pre-treated with NO scavenger PTIO. n=3, *p<0.001 vs. Netrin-1 in Control group. **B)** Netrin-1 increases the proliferative activity of EPCs, while having no effect on EPCs isolated from $DCC^{+/-}$ animals. n=5-6, *p<0.01, **p<0.001 vs. control of $DCC^{+/+}$ EPCs. **C)** EPC apoptosis measured by caspase 3 activity after the treatment of hydrogen peroxide (500 µmol/L, 30 min), showing that netrin-1 preconditioning (100 ng/ml) for 30 min significantly reduced apoptosis while this response was absent in PTIO pretreated cells. n=3, p<0.05 **D)** EPC apoptosis was measured by TUNEL assay after the treatment of hydrogen peroxide (500 µmol/L, 30 min), showing that preconditioning of netrin-1 (100 ng/ml) for 30 min significantly reduced apoptosis. n=4-6, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 vs. Control, ***p<0.001 vs. Netrin-1, $^{\$}$p<0.05, $^{\$\$\$}$p<0.001 v.s. Netrin-1+$H_2O_2$ **E)** Representative pictures of TUNEL assay. Apoptotic cells detected by TUNEL staining is shown in green, whereas total cellular DNA detected by PI staining is shown in red. Grouped quantitative data are shown in panel D).

Netrin-1 induces NO• production and eNOS activation in EPCs

Enhanced endothelial repair is attributed to the enhanced EPC proliferation, viability, and NO•production. This EPC-derived NO•production has been shown to exert antioxidant action and prevent further endothelial injury [22]. Thus, we examined whether NO•production changes in netrin-1-treated EPCs. Figure 2-10A shows significant upregulation of NO•production from netrin-1-treated EPCs compared to non-treated EPCs. Further, we demonstrated that there is an increase in eNOSs1179 phosphorylation in EPCs treated with netrin-1 (Fig. 2-10B). Therefore, netrin-1 promotes EPC function, not only by increasing its number and viability but also by improving eNOS activation and NO• production.

Figure 2-10.

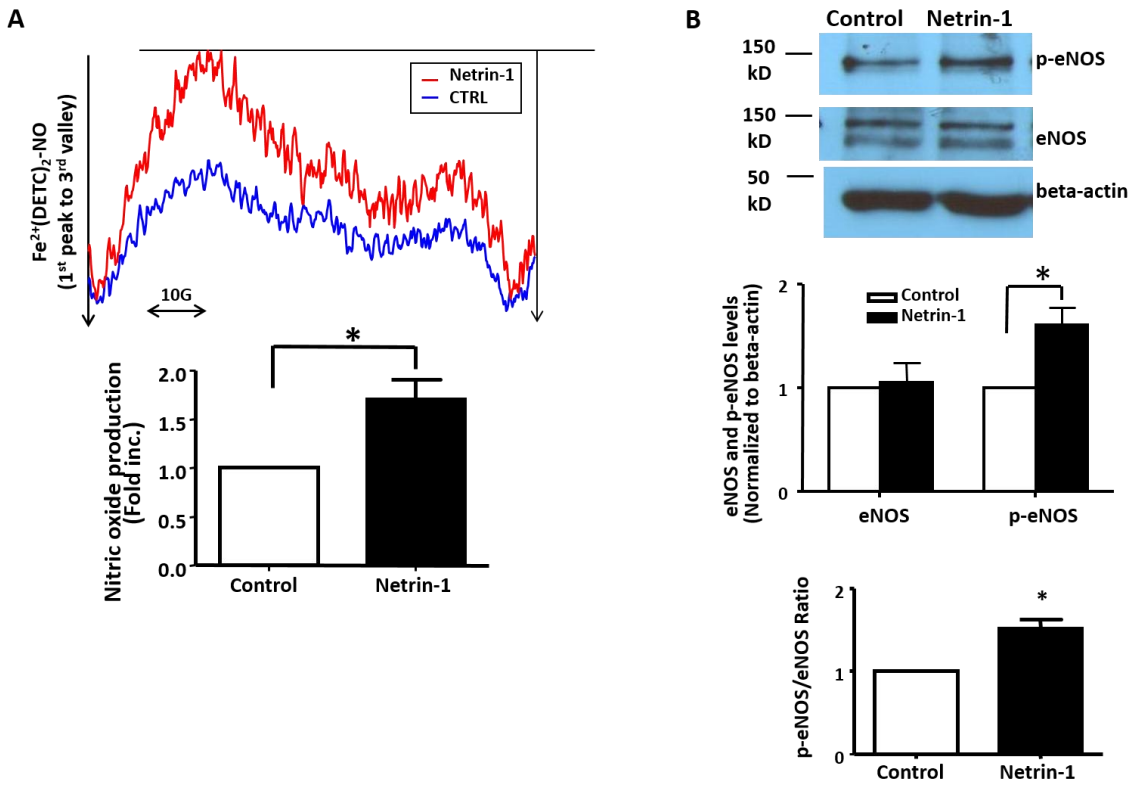


Figure 2-10. Netrin-1 induces NO production and eNOS activation in EPCs. A) NO production from EPCs was measured using ESR. Netrin-1 treatment increased NO production by 70%. n=6, $p<0.05$. **B)** p-eNOS to eNOS ratio (normalized with beta-actin individually) in netrin-1 treated EPCs. Netrin-1 treatment significantly increased the phosphorylation of eNOS at serine 1179. n=3, $p<0.05$

2-4. Discussion

In this work, we examined the vascular protective effects of netrin-1 against neointimal growth induced by wire injury in the femoral artery. Netrin-1 infusion abolished neointimal formation as assessed by ratio of intimal to medial thickness, when compared to untreated controls. This effect was absent in animals lacking the netrin-1 receptor DCC. VSMC proliferation in situ was largely attenuated by netrin-1. VSMC migration, one of the major events in neointimal growth, was inhibited with netrin-1 and mediated by the $NO^{\bullet}$/cGMP/p38 MAPK pathway. Rapid acceleration of EPC homing and re-endothelialization were induced by netrin-1. In separate experiments, we injected netrin-1-treated EPCs to femoral injured animals, which also reduced the ratio of intimal to medial thickness when compared to animals injected with untreated EPCs. We further observed that EPCs treated with netrin-1 had improved proliferative activity, increased resistance to oxidative stress-induced apoptosis, increased eNOS serine 1179 phosphorylation, and elevated $NO^{\bullet}$ production. Overall, these data clearly define a novel and robust effect of netrin-1 serving as a vascular protective agent against neointimal formation/restenosis following endothelial damage. A schematic summary of the mechanisms that are elucidated in this study is shown in Figure. 2-11.

Netrin-1 has been shown in the past to have angiogenic properties [8, 23, 24]. In our laboratory, we have previously shown that netrin-1 enhances endothelial proliferation and migration through a $NO^{\bullet}$-dependent pathway [8]. In this study, we examined whether netrin-1's enhancement of endothelial activity and EPC function can offer protection against neointimal formation in a model that simulates angioplasty. Intriguingly, infusion of netrin-1 resulted in near complete attenuation of neointimal formation, which was absent in animals deficient in DCC. This is even more potent than the overexpression of eNOS, which attenuated neointimal formation by 70% [25]. Protein S-nitrosothiols that release $NO^{\bullet}$ chronically and locally also attenuates neointimal formation after arterial balloon injury in a rabbit model [26]. Combined with our previous notion that relatively modest concentration of netrin-1 is optimally cardioprotective, persistent and modest release of $NO^{\bullet}$ from netrin-1 is particularly vascular protective.

Restenosis is a consequence of a series of events triggered by endothelial damage [2]. Damage to the endothelium result in a loss of $NO^{\bullet}$ production from eNOS, which plays a central role in endothelium-dependent vascular protection [27]. Although VSMCs are quiescent under normal conditions, they re-express mitogenic factors under pathological conditions such as restenosis and atherosclerosis [28]. Restricted levels of $NO^{\bullet}$ promote

VSMC proliferation and migration [29], while inducing vasoconstriction [30]. Migrating VSMCs infiltrate to the intimal area and form the intimal cushion. VSMC migration is provoked by various factors, such as chemotactic cytokines, growth factors, and extracellular matrix components [31]. Our co-culture results demonstrated that cGMP and p38 MAPK are sequentially involved in netrin-1 inhibition of VSMC migration. Previous findings indicate that p38 MAPK mediates VSMC migration in response to platelet-derived growth factor (PDGF) [32] and vascular endothelial growth factor (VEGF) [33]. P38 MAPK has also been shown to decrease mitogenic responses in cardiomyocytes [34]. These disparate responses reflect varied roles of p38 MAPK in different cell types and in response to different stimuli. We further showed that p38 MAPK is downstream of cGMP. The mechanistic details of cGMP signaling to p38 MAPK remain to be elucidated, though it has been suggested to occur in cardiomyocytes [35, 36]. The other two major MAPK pathways, ERK1/2 and JNK, are often studied as pro-proliferative pathways, whereas the p38 MAPK pathway is often associated with anti-proliferative pathways [37]. This might explain the distinct response of p38 MAPK to netrin-1 among those three MAPKs, suggesting that netrin-1 may play a role in inhibiting chemotaxis, rather than DNA synthesis of VSMCs. In addition, loss of $NO^{\bullet}$

inhibition of inflammatory responses in general would contribute to neointimal formation, which can be corrected by infusion of netrin-1 to stimulate $NO^{\bullet}$ production from the post-injury remaining endothelial cells.

The treatment with progenitor or stem-like cells has been shown in the past to reduce neointimal growth after vascular injury [18, 38–40]. In one study that also used the femoral artery injury model, intravenous injection with iPS-cell-derived Flk-1^{+} cells [39] resulted in reduction of neointimal formation when more than 1×10^{6} of the cells were injected. In our study, we injected only 500 EPCs, which had no significant effect on neointimal formation. However, preconditioning of these EPCs with netrin-1 markedly attenuated neointimal formation, which could be attributed to augmentations in proliferative activity, resistance to apoptosis, and $NO^{\bullet}$ production. After the loss of the endothelium due to vascular injury, the timely recovery of the endothelial layer is essential for the restoration of endothelial homeostasis, attributed to the potent inhibitory effects on neointimal formation of endothelium derived $NO^{\bullet}$[25]. Indeed, we observed the rapid acceleration of EPC homing and re-endothelialization in femoral arteries 1 h after endothelial injury and netrin-1 tail vein injection. Furthermore, the number of functional EPCs is important for the proper healing of the endothelium after vascular repair, while reduced EPC number has been observed in

patients with hypertension and coronary artery disease [41]. Oxidative stress develops after vascular injury [42]. Therefore, the effect of netrin-1 in improving the resistance to oxidative stress-induced apoptosis of EPCs would facilitate survival of the EPCs to enhance repair. Taken together, our data also support the use of limited numbers of circulating EPCs for anti-restenosis therapy.

In conclusion, these data establish netrin-1 as a potent vascular protective agent against neointimal growth following femoral wire injury of the endothelium. This effect of netrin1 is dependent on the receptor DCC for either netrin-1 infusion or treatment with netrin-1 preconditioned EPCs. The protection is mediated by $NO^{\bullet}$/cGMP/p38 MAPK-dependent inhibition of VSMC migration, and augmented functions of EPCs in terms of enhanced proliferative activity, increased resistance to oxidative stress-induced apoptosis, and increased eNOS phosphorylation and $NO^{\bullet}$ production. These findings may promote usage of netrin-1 and netrin-1 preconditioned EPCs as novel therapeutics treating post-angioplasty restenosis, which would be highly significant in the management of myocardial infarction at bedside.

Figure 2-11.

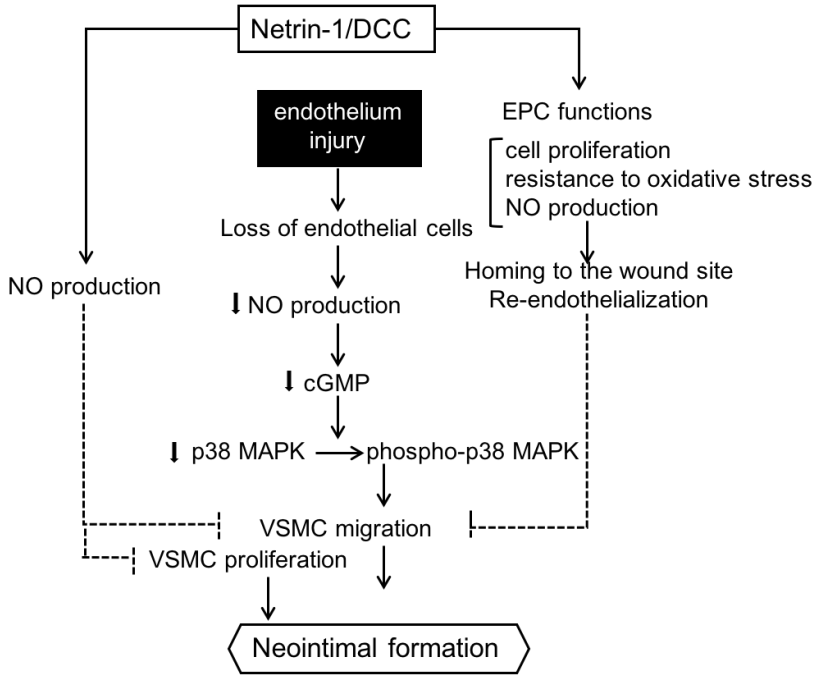


Figure 2-11. Schematic summary of mechanistic insights of netrin-1 inhibition of restenosis. Endothelium injury causes restenosis by reducing NO production from the loss of the endothelial cells and subsequently removing its inhibition on VSMC migration. Netrin-1 treatment inhibits VSMC migration via NO/cGMP/p38MAPK. *In situ* VSMC proliferation was also inhibited by netrin-1 perfusion. In addition, netrin-1 augments functions of EPCs, including proliferation and resistance to oxidative stress, facilitating timely homing of EPCs to the wound site. These downstream pathways contribute to marked inhibition of netrin-1 on neointimal formation post endothelial injury.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

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3. Protective effect of netrin-1 in atherosclerosis

The protective effects of netrin-1 in the settings of pathophysiological processes of angiogenesis and neointimal formation are discussed in the previous chapters. As a next step, we tested whether netrin-1 is also effective in the prevention of atherosclerosis. Here we used apolipoprotein E null ($apoE^{-/-}$) mice with high fat food feeding. ApoE is a member of plasma lipoproteins which transport lipids to various cells and tissues. It is important in cholesterol metabolism. $ApoE^{-/-}$ mice spontaneously develop atherosclerotic lesions with a normal chow diet, and the lesion develop even more robustly with a western-type diet, leading to a rupture of plaques[1]. Although the pathogenesis remains incompletely understood, its certainty of atherogenic activity by affecting lipid metabolism and distribution made it one of the strongest models to study atherosclerosis. Indeed, $apoE^{-/-}$ mice are used in a great amount of research in testing new drug target against atherosclerosis.

3-1. Introduction

Atherosclerosis is a chronic arterial disease, which is one of the most prevalent cardiovascular disorders especially in developed countries. The pathogenesis of atherosclerosis starts with endothelial damage due to hyperlipidemia. The EC damage reduces $NO^{\bullet}$ bioavailability often consequent to increased ROS production. The so called "malignant" lipoprotein, low density lipoprotein (LDL) is oxidized (ox-LDL) in such environment. ECs are activated by ox-LDL intake, which promotes immune cell attraction and leading to increased intake of macrophages that differentiated from monocytes. The macrophage and ox-LDL further react together and accumulate as foam cells in subendothelial region, which is recognized as fatty streak [2]. Fatty streaks in arterial walls contribute gradual development of atheroma and plaques [3]. The plaque can be ruptured and form a thrombosis, which would directly lead to a sudden death from heart attack or stroke. Not to mention its huge impact on mortality, the tremendous economic burden has urged scientists to develop more effective therapeutics. According to National Heart, Lung, and Blood Institute, options on atherosclerosis treatment are thus far limited to medications to lower the cholesterol levels, and blood pressure and blood glucose levels, and prevention of

inflammation and blood clots. In addition, people with high risk factors are recommended to have a healthier lifestyle, but medical procedures and surgery such as coronary angioplasty, bypass grafting and endarterectomy, are required in some severe cases.

As indicated above, LDLs, the main blood cholesterol carrier, are oxidized in athero-prone environment due to accumulated ROS. Ox-LDLs are the causal substances in atherosclerosis progression [4]. They damage various cells including endothelial cells therefore inducing endothelial dysfunction. Reduced bioavailability of $NO^{\bullet}$ impairs vasodilation, often consequent to pro-inflammatory and pro-thrombotic states. A well-known study from Heitzer T et al. demonstrated endothelial dysfunction and defective vasodilatation in chronic smokers, who have elevated oxLDLs [5].Moreover, oxLDLs induce apoptosis as well as autophagy of endothelial cells both resulting in endoplasmic reticulum (ER) stress [6]. Hence, endothelial cells play a key protective role against atherogenesis.

As discussed in the previous chapters, netrin-1 protects endothelial cells by activating DCC/ERK1/2/eNOS pathway [7]. The function of endothelial progenitor cells (EPC) are also promoted by netrin-1 [8]. We found these effects presented by netrin-1 mediate accelerated repair of endothelial injury [8]. In this chapter, we subsequently investigated whether netrin-1 can be a therapeutic option for atherosclerosis.

It is important to note that endothelial dysfunction and inflammatory responses are major driving forces in the pathogenesis of atherosclerosis[9]. The inflammatory responses are activated as a consequence of endothelial dysfunction. Platelet aggregation, which would further generate inflammatory cytokines, is attenuated by physiological levels of $NO^{\bullet}$ from eNOS activation. Thus, inflammatory activation is induced due to endothelial dysfunction in atherosclerosis. When platelets are activated by attaching to the arterial wall, it also attracts monocytes to form platelet-monocyte aggregation. Monocytes represent a major portion of immune response, contributing to plaque progression during atherogenesis[10]. Interestingly, monocytes express UNC5B, the receptor of netrin-1, implying the expression of netrin-1 by endothelial cells may inhibit inflammation by repulsing monocyte recruitment [11].

Hence, we hypothesized that netrin-1 treatment in atherogenic animals alleviate or delay the progress of atherosclerosis, via endothelial protection and inhibition of monocyte adhesion. Atherogenic mice are created by feeding $apoE^{-/-}$ mice with high fat for 16 weeks. Netrin-1 was infused into the animals for the entire time of high fat diet feeding. The pathological responses were evaluated by measurements of the increase in body weight, plasma lipid profiles, and histological analyses of aortas. Additional investigation on monocyte modification by netrin-1 was also conducted.

3-2. Methods

Reagents

Mouse recombinant netrin-1 was purchased from R&D Systems (Minneapolis, MN). Assay kit for cholesterol, triglyceride and HDL-cholesterol were all purchased from Pointe Scientific (Canton, MI). Standard solutions for triglyceride and HDL-cholesterol were also purchased from Pointe Scientific. Standards for cholesterol assays were prepared using the powder obtained from Sigma-Aldrich (St Louis, MO). The antibody used for macrophage staining, Rat Anti-Mouse CD107b (Mac3) was purchased from BD Biosciences (San Jose, CA). M1 macrophage marker IL-1β Mouse monoclonal antibody and M2 macrophage marker Arginase-1 Rabbit monoclonal antibody were purchased from Cell Signaling Technology (Danvers, MA). 4'6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) solution was from Invitrogen (Carlsbad, CA). Histopaque 1083 (Sigma-Aldrich, St Louis, MO) and calcein AM (Calbiochem, Germany) were prepared for monocyte isolation and labeling. Two different antibodies for UNC5B were purchased from Abcam (Cambridge, MA) and Enzo Life Sciences (Farmingdale, NY).

Animals

All animal procedures were approved by the Institutional Animal Care and Usage Committee at the University of California, Los Angeles (UCLA). Breeders of apoE$^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, ME, Strain B6.129P2-Apoe^{tmlUnc}/J) and bred in house till experimental use. Twelve- to fourteen-weeks old male animals were infused with or without netrin-1 (15 ng/day) a day in advance to the commencement of high fat diet (HFD / 42% fat; Harlan Laboratories, Madison, WI) for 16 weeks.

Netrin-1 infusion using osmotic pumps

Netrin-1 infusion was made using osmotic pumps. In order to complete 16 weeks treatment, osmotic pumps were replaced twice during the HFD. Two 6-week pumps and a 4-week pump were used (ALZET, Cupertino, CA) per animal. Animals were anesthetized with isoflurane in a closed chamber and moved onto surgical stage where an inhalation anesthesia mask is provided. While supplying 95%/5% of O_2/CO_2 mixed with 1.5% isoflurane, hair at lower posterior neck area was removed and disinfected using ethanol and iodine solusion. A small incision was made at this area and 6-week or 4-week osmotic pump was inserted

subcutaneously.

Tissue collection

After 16-week of HFD feeding, animals were weighed and sacrificed by euthanizing with CO_2. Heparin (0.1 mL) was injected via left ventricle and left it to circulate for a minute and blood was collected slowly from right ventricle using 14G needle. The collected blood was used to analyze lipid profile. The animals were then perfused with ice-cold PBS to flush out remaining blood in the body. The entire lobes of liver was removed to record its weight for each animal. The aorta connecting the heart was excised immediately from the body and the connective tissue was cleaned under a surgical microscope. The upper quarter of the heart including the root of aorta was used for histological analysis because tricuspid/aortic root tends to show atherosclerotic lesion most frequently compare to the other part of aorta. Rest of the part of aorta including ascending aorta, aortic arch, descending aorta and iliac artery branch point, was harvested and opened en face for Oil Red-O staining.

Plasma lipid profile measurement

The blood collected from right ventricle was placed in a 1.5 mL centrifuge tube and centrifuged at 2000 rpm for 10 min. The semi-transparent plasma phase was replaced into a new centrifuge tube and used for each assay. Levels of cholesterol, triglyceride and HDL-cholesterol were measured based on colorimetric change per manufacturer's instruction. The absorbance at 500 nm was detected using Bio-Tek plate reader.

Oil Red-O staining on aorta dissected en face

Three percent of oil red-O solution was prepared in 2-propanol and the reagent was heated to 56℃for 1 hr. The oil red-O reagent was diluted to 1.8 % by mixing with water and filtered with a 0.22 μm syringe filter. Aorta opened en face was rinsed once with 0.5 % triton including PBS (PBST) and then stained with working oil red-O solution for 30 min at room temperature. Following the staining, aorta was washed with 2-propanol for a min and returned to PBST for additional wash. The aorta image was obtained under the Nikon E600 microscope and the stained lesion area was calculated using ImageJ.

Aortic root histological analysis

The upper quarter of heart including aortic root area was sliced and collected into 4% of paraformaldehyde for O/N for fixation. Tissues were displaced in 10% sucrose for at least a few hour prior to being embedded in paraffin for sectioning at 5 μm. The paraffin section slicing and H&E staining was performed using the standard protocols in the Translational Pathology Core Laboratory Core Facility at UCLA. The slices tissue sections were also used for macrophage staining using anti-CD107b (Mac 3) antibody. All of the stainings were performed double-blindedly.

Monocyte isolation

Monocytes were isolated from the bone marrow of 6 to 8-week old male C57BL/6 mice. Femur and tibia bones were harvested and cleaned, and ice-cold PBS was flushed into the bones using insulin syringe. Histopaque 1083 was used to separate mononuclear cells by centrifuging at 400 xg for exactly 30 min at room temperature. The middle opaque layer was carefully replaced and washed with RPMI1640.

Monocyte-endothelial adhesion assay

Bovine aortic endothelial cells (BAECs) were cultured in M199 medium on a 96-well plate until more than 80% confluent. On the day of the assay, 100 ng/mL of TNFα was added to BAECs while harvesting monocytes from the bone marrow. The concentration of 1.0 x 10^6 cells/mL of isolated monocytes were labeled with calcein AM. In the meanwhile, BAECs are treated with or without PTIO for 30 min. After 30 min of labeling, the monocytes were washed twice with medium and treated with or without anti-UNC5B antibody (1 μg) to block UNC5B receptor from binding with netrin-1. At the same time, netrin-1 (100 ng/mL) was added to BAECs. Both cells underwent another 30 min of incubation. At the end of the incubation, the medium of BAECs were removed and washed with warm PBS once. Then monocytes in 100 μL PBS were overlaid on top of BAECs and co-cultured for 30 min. Cells were gently washed with PBS twice to remove unattached cells. The level of calcein AM labeled monocytes adhere to BAECs were detected by reading at excitation/emission: 485/528 nm in a Bio-Tek fluorescence plate reader.

UNC5B detection in monocytes

In order to observe the cleavage of UNC5B in response to netrin-1 binding, monocytes were treated with or without netrin-1 for 30 min. After the treatment, the pellet of cells were lysed,

and subjected to detection of intact UNC5B and cleaved UNC5B protein levels by Western blotting per standard protocols using 7.5 % SDS/PAGE and nitrocellulose membranes. Antibody from Abcam (#ab139643/ 1:500 dilution) was used to detect intact form of UNC5B, whereas the antibody from Enzo Life Sciences (#ALX-804-846-C100/ 1:500 dilution) was applied for detection of cleaved-UNC5B. The ratio of expression levels of cleaved-/ intact-UNC5B was calculated.

3-3. Results

Netrin-1 infusion attenuated development of atherosclerosis

In order to create an atherosclerotic model, netrin-1 was infused one day in advance to HFD commencement in apoE$^{-/-}$ mice and continued for 16 weeks for the experimental group (Figure 3-1A), whereas control group (CTRL) was prepared without netrin-1 infusion. After 16 weeks of HFD, the appearance of two groups of mice are very different. As shown in Figure 3-1B, the body weight increase in CTRL was extremely apparent, while Netrin-1 group stayed unchanged. The anatomical difference was mostly seen in both fat amount and liver hypertrophy, which simulating patients with higher risk of atherosclerosis. Further to visualize atherosclerotic lesion in aortas, oil red-O staining was performed and netrin-1 significantly decreased the occurrence of lesion formation (Figure 3-1C, D). Subsequently, plasma lipid profile was also examined. High levels of low-density lipoprotein cholesterol (LDL) and triglycerides, and a low level of high-density lipoprotein cholesterol (HDL) are the symbolic profile in atherosclerosis patients [12]. Figure 3-2A-C showed netrin-1 lowered the levels of total cholesterol and triglyceride significantly, while HDL-cholesterol remained to be similar. Thus, apoE$^{-/-}$with HFD created the model of human atherosclerosis pathology, and netrin-1 successfully alleviated the symptoms including body fat mass, liver hypertrophy,

lesion formation in aortas, and blood lipid profiles.

Figure 3-1.

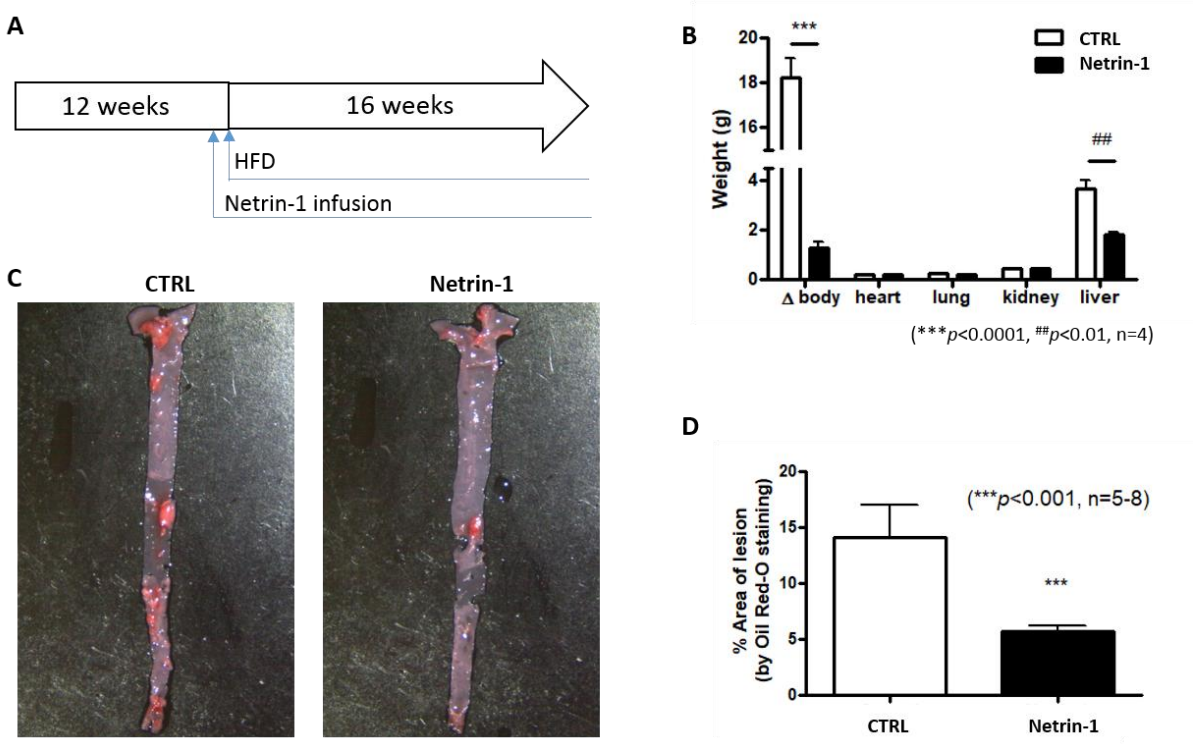


Figure 3-1. Netrin-1 infusion prevented body weight increase and alleviated lesion formation in apoE$^{-/-}$ mice fed high fat diet. ApoE$^{-/-}$ mice were fed with high fat diet (42% fat) for 16 weeks, with or without netrin-1 infusion (15 ng/day). There were significant differences in body weight change and liver size **(A)**. Oil red-O staining was performed to observe lesion formation. As is obvious, netrin-1 significantly abrogated lesion formation **(B&C)**.

Figure 3-2.

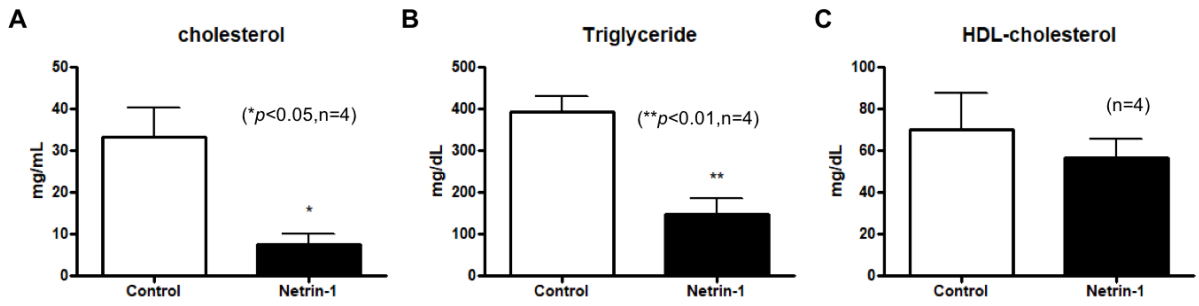


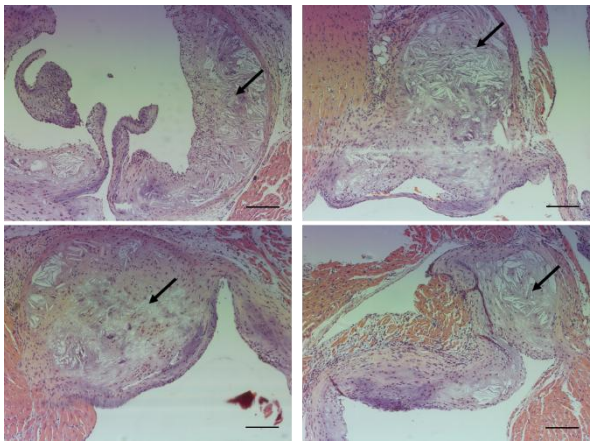
Figure 3-2. Netrin-1 infusion improved plasma lipid profile in apoE$^{-/-}$ mice fed high fat diet. Levels of plasma cholesterol and triglyceride were robustly decreased with netrin-1 infusion, while there was no difference in HDL-cholesterol levels.

Netrin-1 infusion decreased fatty streak accumulation at aortic root

Hypercholesterolemia induced by apoE knockout and accelerated by HFD, results in a formation of fatty streak. Which is known as first grossly visible lesion to be formed during the development of atherosclerosis[3]. Triggered by fatty streak accumulation, subsequent vicious cycle of pathogenesis such as fibrosis and plaque rupture follows. The fatty streak is most frequently seen at the root of aorta which can be identified by the unique structure of tricuspid valve. Therefore, we screened the aortic root of both CTRL and Netrin-1 infused groups (Figure 3-3). The result showed 100 % chance for CTRL to develop huge fatty streak. This is reasonable since apoE$^{-/-}$ mice develop hyperlipidemia and atherosclerosis even with normal chow diet as they age [13]. On the other hand, there were only three out of seven animals (=43%) with netrin-1 infusion developed fatty streak. Moreover, the fatty streak lesion in netrin-1 treated animals were relatively smaller. Hence, netrin-1 decreased fatty streak incidence in aortic root.

Figure 3-3

A



B

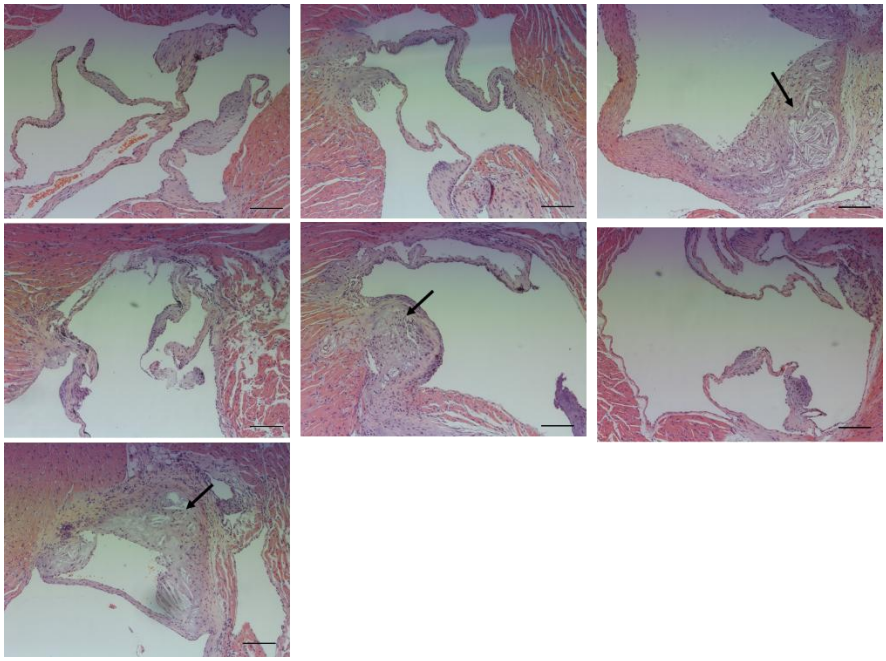


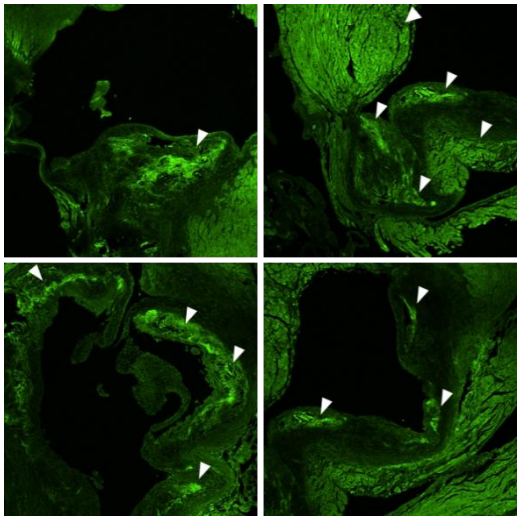
Figure 3-3. Netrin-1 infusion decreased accumulation of fatty streak at aortic root in apoE$^{-/-}$ mice fed high fat diet. Four out of four animals (=100%) of CTRL **(A)**, while only three out of seven animals (=43%) with netrin-1 infusion **(B)** developed fatty streak. Black arrows indicate fatty streaks.

Netrin-1 infusion prevented macrophage infiltration at the aortic root

Under atherosclerotic environment, circulating monocytes are retained to endothelium and differentiated into macrophages in the subendothelial region. The differentiated macrophages react with modified LDLs such as ox-LDLs to form foam cells. The foam cells capture various immune cells such as T-cells, dendritic cells, and mast cells [2]. This reaction further recruits more inflammatory cells and modified LDLs, leading the initiation and fatty streak phase of atherosclerotic lesions [2]. Therefore, macrophage accumulation can be evaluated as the earliest sign of atherogenesis by immunochemical staining. Figure 3-4 showed apparent difference in amount of Mac-3 (CD107) positive cell between control and netrin-1 infused animals. This result was consistent to the fatty streak formation data showed in Figure 3-3, hence, it once again confirmed alleviated development of atherosclerosis at the early stage, by netrin-1 treatment.

Figure 3-4.

A



B

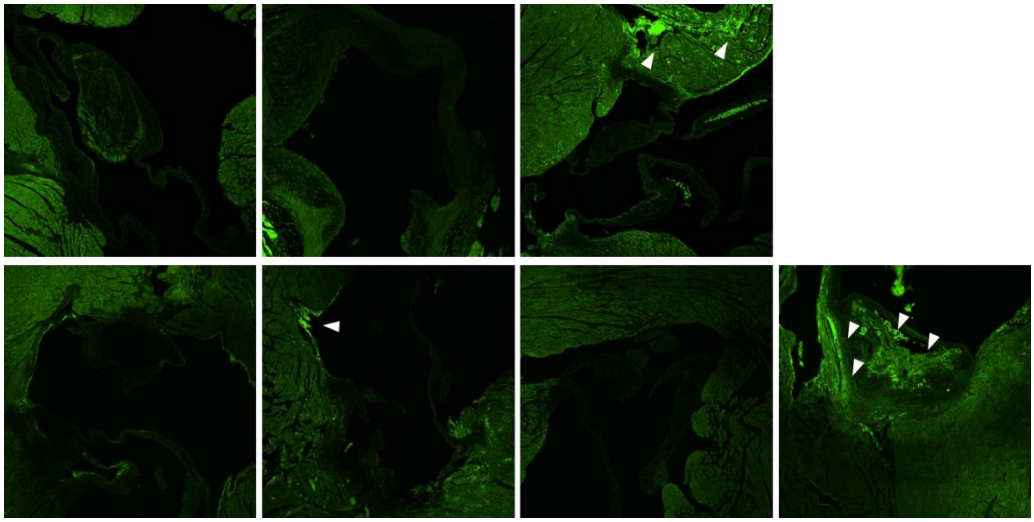


Figure 3-4. Netrin-1 infusion reduced macrophage infiltration at the aortic root.

Immunohisto-chemistry using anti-Mac3 (CD107) antibody was performed to visualize macrophage accumulation. The most intense responsive areas of aortic roots were selected double-blindedly. CTRL animals **(A)** had stronger signals compare to netrin-1 infused animals **(B)**. White arrowheads indicate Mac3 positive cells.

Netrin-1 infusion inhibited monocyte adhesion to endothelial cells via UNC5B

Monocytes, the major source of inflammatory response in atherosclerosis, express UNC5B as a dominant receptor of netrin-1 [11]. UNC5B receptor is known to be a repulsive receptor of netrin-1; thus we hypothesized that monocytes expressing UNC5B are repulsed by netrin-1 expressed in endothelial cells, contributing the alleviation of inflammatory response in atherosclerosis. Thus monocyte-EC adhesion assay was performed. Figure 3-5A showed a 23% inhibition of adhesion by treating ECs with netrin-1. However, if monocytes were pre-treated with UNC5B antibody to mask the receptor, netrin-1 treatment on EC had no effect on monocyte adhesion. Although further analysis is needed, this result suggests that UNC5B is mediating the anti-adhesion effect in the presence of netrin-1.

It is also known that when netrin-1 binds to UNC5B, the intracellular death domain of UNC5B is cleaved and the cell expressing UNC5B proceed to apoptosis [14]. Therefore, the cleavage of UNC5B was detected in response to netrin-1 treatment in monocytes. The result showed that netrin-1 significantly increased the expression of cleaved-UNC5B (Figure 3-5B), indicating that netrin-1 binding did increase death domain activation in monocytes.

Hence, it is reasonable to assume that anti-inflammatory effect of netrin-1 is at least partially mediated by anti-attraction and pro-apoptosis of monocytes via UNC5B expression.

Figure 3-5.

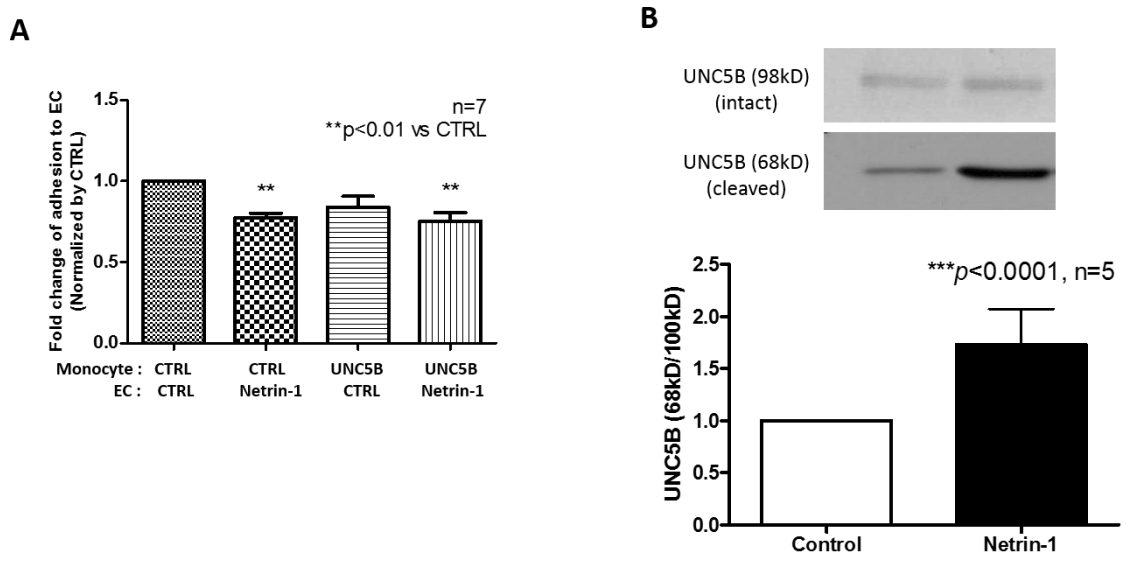


Figure 3-5. Netrin-1 infusion attenuated monocyte adhesion to endothelium via UNC5B.

The monocytes-EC adhesion assay was performed using Calcein-AM labeling on monocytes **(A)**. The result shows netrin-1 treatment on ECs inhibited monocyte adhesion, but the effect was reduced by UNC5B antibody pre-treatment on monocytes. (n=7, **p<0.01 vs CTRL) Shown in (B) is western blot analysis of monocyte in response to netrin-1 treatment. UNC5B cleavage level was increased in monocytes in the presence of netrin-1 (n=5, ***p<0.0001).

3-4. Discussion

Atherosclerosis is considered as a chronic inflammatory disease primarily caused by hyperlipidemia. It has long been known that hyperlipidemia environment caused endothelial dysfunction shown by reduced $NO^{\bullet}$ bioavailability [15]. A recent study showed major involvement of endothelial cells in cholesterol crystal formation at the subendothelial region [16], suggesting the rather important contribution of ECs in plaque formation than inflammatory cells especially at the earlier stage of atherogenesis. Therefore, we assumed the protective effect of netrin-1 in ECs and EPCs mediated by $NO^{\bullet}$ production would alleviate atherosclerosis progression. Our results showed abolished weight increase and liver hypertrophy, and reduced lesion formation by netrin-1 infusion. The plasma lipid profile was also improved by netrin-1. While total cholesterol and triglyceride levels were drastically reduced in netrin-1 infused animals, HDL-cholesterol did not change. HDL-cholesterol removes unesterified cholesterol from peripheral cells and delivers to hepatic cells, known as reverse cholesterol transport [17][18]. Therefore it has anti-atherosclerotic, anti-inflammatory, and endothelial protective effect. Although our HDL-cholesterol measurement in CTRL animals was as high as netrin-1 infused animals, the ratio of HDL- versus total-cholesterol is much lower in CTRL group. Hence, the plasma lipid profiles of CTRL and netrin-1 groups

successfully simulated atherosclerosis and healthy subjects, respectively.

Another important finding from our study is that netrin-1 inhibited monocyte adhesion to ECs, and increased UNC5B cleavage in monocytes. These results indicate that netrin-1 repelled monocytes from endothelium, and increased monocyte apoptosis. Although further studies are warranted, it may be reasonable to assume netrin-1 binding to UNC5B in monocyte induced cleavage of death domain of UNC5B and activated apoptosis [14]. This implies that netrin-1 may prevent plaque rupture, meaning that netrin-1 may be able to reverse the earliest stage of atherosclerosis lesion. This is consistent to our finding on reduced macrophage accumulation in netrin-1 infused animals. However, future study is definitely needed, for example, by utilizing UNC5B mutant animals to confirm an intermediate role of UNC5B is monocyte-dependent protective mechanisms of netrin-1 on atherogenesis.

In summary, our study demonstrated netrin-1's protective effect against pathogenesis of atherosclerosis is at least partially mediated by recovering endothelial function via restored $NO^{\bullet}$ production and also by inhibiting monocyte activation. We believe this study will shed new light on netrin-1's potential in preventive medicine. In view of the high prevalence of atherosclerotic cardiovascular diseases, our findings might of be high translational potential in promoting novel therapeutics.

3-5. References

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4. Netrin-1 promotes cell cycling of endothelial cells

In previous chapters, the protective effect of netrin-1 was demonstrated using in vivo models that simulating human vascular diseases. Those positive outcomes of protective effects were mediated by $NO^{\bullet}$ production from both endothelial cells or endothelial progenitor cells. Netrin-1 activate eNOS via ERK activity when it binds to DCC receptor that is dominantly expressed in the ECs, which promotes mitogenic activity of ECs [1]. However, the mechanistic details as to how netrin-1 induces endothelial cell proliferation remain unknown. In this chapter, we investigated the effects of netrin-1 on endothelial cell cycling and proliferation. The phosphorylation of PKC (pan, ser660), which is known to induce $p21^{waf1/cip}$, as well as the expressionsof $p27^{kip1}$, $p21^{waf1/cip}$, and phosphorylation of Retinoblastoma (Rb) were examined for cell cycle modification made by netrin-1 treatment in ECs.

4-1. Introduction

Previously, we have demonstrated that netrin-1 promotes angiogenesis in endothelial cells through ERK1/2 dependent $NO^{\bullet}$ production following DCC activation [1]. Wound healing orchestrated by cell proliferation and migration was accelerated by netrin-1 treatment[1]. However, the underlying mechanisms whereby netrin-1 modulates endothelial cell proliferation remain unclear. The wound repair process demands complex and coordinated molecular signaling of endothelial cells. The cell cycle not only plays an important role for these signaling but also directly influences multiple cell processes, including DNA repair, apoptosis, cell proliferation and cell migration[2].

Cell cycle is tightly regulated by transcription factors, cyclins, cyclin dependent kinases (CDKs), and their inhibitors (CKIs). In particular, the retinoblastoma tumor suppressor protein (Rb) acts as the key switch in modulating the mammalian cell proliferation by inhibiting the transition of G1-S phase [3]. While binding of Rb to E2F family transcription factors suppresses their transcriptional activity, phosphorylation of Rb mediated by cyclin dependent kinase (CDK)-cyclin complexes causes conformational changes to release E2Fs. Then expressions of genes required for progression towards S phase, including cyclin E and cyclin D are facilitated by E2Fs[4]. In addition, Rb protein activity during the

cell cycle can be modulated by cyclin expression, phosphatase activity, and induction of cyclin-dependent kinase inhibitors (CKIs), which are members of the CIP/KIP family (p21, p27, and p57), and INK4 family (p15, p16, p18, and p19). Furthermore, ERK1/2 signaling is involved in G1-S transition cell cycle progression via different mechanisms including regulation of cyclin D, $p27^{kip1}$ and $p21^{waf1/cip}$[5]. Given the ERK1/2 activation of netrin-1 in endothelial cell as shown previously [1] and the role of Rb in cell cycle, netrin-1 might be involved in Rb mediated cell cycle regulation for endothelial angiogenesis.

Therefore, we investigated the effect of netrin-1 on cell cycle regulation to elucidate the mechanisms for proliferation of endothelial cells (ECs). In this study, we found that netrin-1 mediates the phosphorylation of Rb, which is attributed to downregulation of $p27^{kip1}$ and $p21^{waf1/cip}$, and subsequently resulting in enhanced cell cycle progression of G1-S transition. However, all these responses were fairly mild, which implies low risk of cancer angiogenesis development. In conclusion, Rb dependent cell cycle regulation by netrin-1 might contribute to its known angiogenic effect in EC mildly but significantly.

4-2. Methods

Reagents

Recombinant mouse netrin-1 was purchased from R&D Systems. Primary antibodies used for western blot are as follows; Rb (#9309), p-Rb (#9308), p27 Kip (#2552), p-PKC (#9371) were all purchased from Cell Signaling, p21 was from BD Pharmingen (#556431), beta-actin was from Sigma (#A2066). HRP conjugated Goat Anti-Rabbit IgG and Goat Anti-Mouse IgG for secondary antibodies were purchased from Bio-Rad.

Cell culture and transfection

Bovine aortic endothelial cells (BAECs, Cell systems, Kirkland, WA) of passages 4-6 were cultured in Media 199 (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) to confluence and starved in 5% FBS contained media overnight before experiments. Some cells were exposed to 100 ng/ml of netrin-1 for desired time course. Proliferating BAECs at 70-80% confluence were transfected with 25 nmol/L Rb1 siRNA (QIAGEN, #SI00007112) using Lipofectamine RNA iMAX (Invitrogen). Culture media was changed after 48 hrs of transfection and subjected to each experiment. Scrambled siRNA was transfected as control (indicated as CT siRNA). The transfection was confirmed by detecting both Rb and phospho-

Rb using western blot.

Cell proliferation assay

Cell proliferation with or without Rb siRNA transfection was assessed using cell counting method as indicated previously[6]. BAECs were seeded at an initial density of $1.0x10^5$ cells/well in 24-well plates and transfected with Rb siRNA as indicated above. Culture medium was changed after 48 hrs of transfection and the cells were treated with or without netrin-1 (100 ng/ml) for additional 24 hrs. Then the cells in each well were trypsinized and counted using a hemocytometer.

Propidium iodide staining for cell cycle analysis

Cell cycle analysis was performed by measuring DNA content in cell using a flow cytometry in core facility of UCLA. Cells were starved with M199 media containing 5 % FBS for 16 hrs and then incubated in presence or absence of 100 ng/ml of netrin-1 for additional 24 hrs. After treatment for 24 hrs, cells were trypsinized and $1X10^6$ cells were used for fixation with 1 ml of chilled 70 % ethanol. The DNA from cells was subsequently stained with 1 ml of

hypotonic staining buffer (0.1 mg/ml of propidium iodide, 0.02 mg/ml of RNAse, 0.3 % Triton X-100, and 1 mg/ml sodium citrate) for 15 min at 37°C. Stained samples were used for flow cytometry analysis using a Becton Dickinson FACScan analytic flow cytometer (Becton Dickinson, Mountain View, CA).

Western blot analysis for cell cycle regulating proteins

After 24 hrs starvation (with 0.5% FBS containing media), the cells were stimulated with or without netrin-1 (100 ng/ml) and collected by scraping the bottom of culture dishes after 1 hr and 6 hrs. The cells were also pre-treated with Gö 6983 (10nmol/L, Sigma #G1918), a PKC inhibitor, for 30 min prior to 6 hr-netrin-1 treatment. Proteins were extracted from the collected cells and separated by SDS-PAGE to determine relative abundance of the cell cycle regulation related proteins: p21, p27, p-PKC, p-Rb. The western blot analyses were performed as described in previous studies[7]. The concentrations of antibodies used are as following: p21 (1:200), p27 (1:1000), p-PKC (1:1000), and p-Rb (1:200). Beta-actin (1:10000) was used as internal control.

4-3. Results

Netrin-1 significantly promotes cell proliferation and G1/S transition

Our previous data demonstrated that netrin-1 accelerates endothelial cell growth in aortic disc and wound closure, which reflects coordinated action of migration and proliferation. This angiogenic effect of netrin-1 was mediated by ERK1/2 dependent NO production. To elucidate the underlying mechanism of angiogenesis by netrin-1, we first checked whether netrin-1 is capable of promoting endothelial proliferation and cell cycle progression. As shown in figure 4-1A, cell proliferation was significantly increased by netrin-1 treatment (1.30±0.04 fold increase compared to control). Further, in order to examine whether the increase of proliferation is due to cell cycle progression, cell cycle was assessed by flow cytometry to measure cellular DNA content. As shown in figure 4-1B, percentage of S phase was increased in netrin-1 treated endothelial cell (7.0±0.37 vs 8.8 ± 0.48, control vs netrin-1, respectively). Thus, the data suggests the angiogenic effect of netrin-1 is mediated by acceleration of cell cycle progression of the endothelial cells.

Figure 4-1.

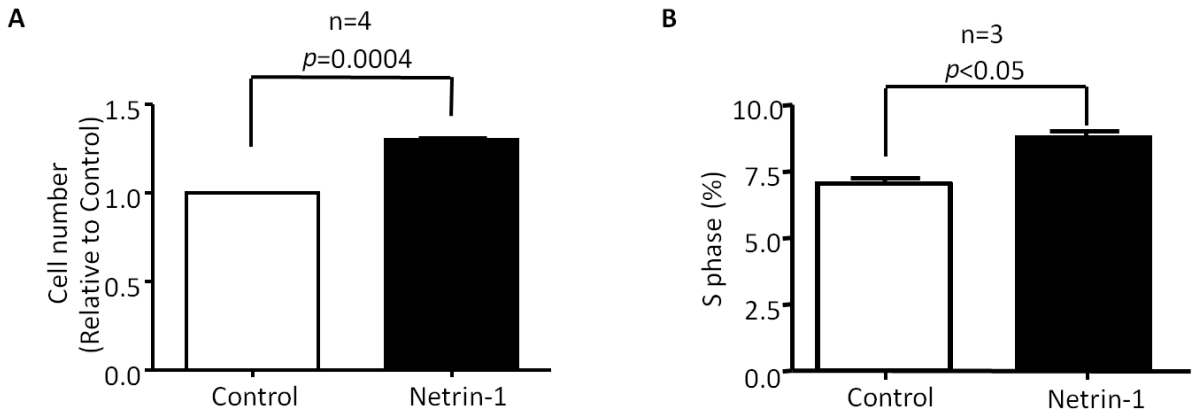

Figure 4-1. Netrin-1 increased cell proliferation and G1 to S phase transition in cultured aortic endothelial cells. Bovine aortic endothelial cells (BAECs) were treated with netrin-1 at 100 ng/mL for 24 hrs prior to analyses. **A)** Cell proliferation was assessed by cell counting with a hemocytometer. Data indicate significant elevation of BAEC proliferation by netrin-1 treatment (n=4, p=0.0004). **B)** Cell cycle was analyzed by PI staining along with flow cytometry (n=3, *p<0.05).

Netrin-1 promotion of BAEC proliferation is mediated by Rb

Phosphorylation of Rb is the key step for cells to progress into S phase, in which cell undergoes chromosome duplication. Therefore, we investigated the expression level of phospho-Rb at 1 and 6 hr time points with or without netrin-1 treatment in BAECs. A significant increase of phospho-Rb was seen at the presence of netrin-1 at 6 hrs (figure 4-2A). In order to further confirm that netrin-1 can promote EC mitosis by modulating cell cycle progression, we interfered Rb expression using siRNA technique. While netrin-1 significantly increased cell proliferation in control siRNA transfected BAECs, it was diminished by Rb-siRNA transfection into BAECs (figure 4-2C). The transfection efficiency is shown in figure 4-2B.

Figure 4-2.

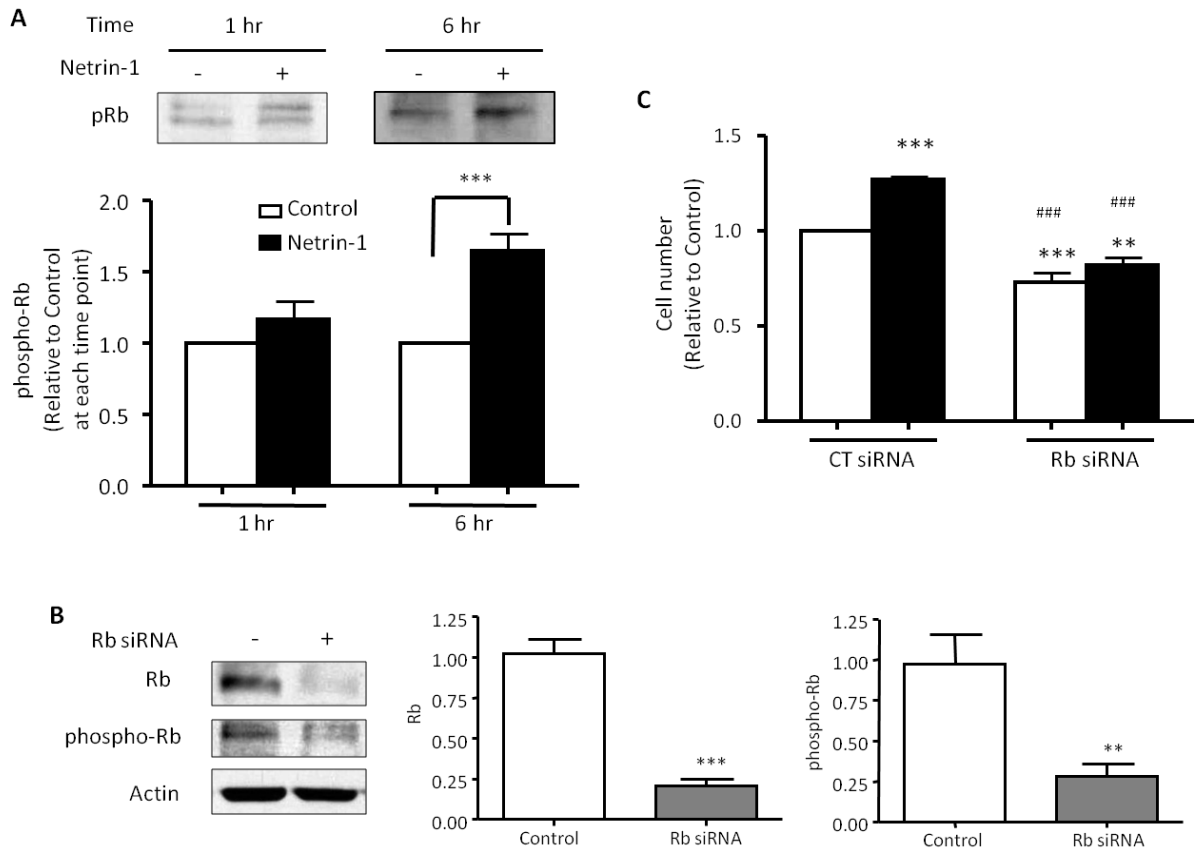

Figure 4-2. Netrin-1 induced cell proliferation is mediated by Rb phosphorylation. A) Phospho-Rb expression in BAEC was increased significantly at 6 hrs post-treatment with netrin-1 (***p<0.001, n=7-8). **B), C)** Cells were transfected with scrambled siRNA or Rb siRNA. Transfection efficiency was assessed by detecting protein expression of Rb (n=6-10, ****p*<0.0001) and phospho-Rb (n=6-10, ***p*<0.001) with western blot **(B)**. Transfected cells were treated with or without netrin-1 for 24 hrs and cell proliferation was measured using cell counting method as described. (n=3, ***p*<0.01, ****p*<0.001 vs Control of CT siRNA, ###*p*<0.001 vs Netrin-1 of CT siRNA) **(C)**.

Rb, p27, p21 and PKC mediates netrin-1 induced cell cycle progression

To delineate the signaling events of cell cycle progression by netrin-1, modulations of phosphorylation of Rb, the key player of G1/S, were examined. As shown in figure 4-2A and 4-3B, phosphorylation of Rb was significantly increased by netrin-1 treatment (1.65±0.13 folds). The phosphorylation of Rb is controlled by CDK2. When cells are quiescent, accumulation of CDK inhibitors (CDKI) can be observed. In particular, p27 inhibits the cyclin E/CDK2 complex, causing the cell cycle arrest in G1 phase[8]. In this study, netrin-1 treatment for 6 hr decreased the expression of p27 as shown in figure 4-3C (0.59 ±0.08 folds), indicating the CDK2 activation by netrin-1. The similar downregulation of a major CDKI p21 also was observed in netrin-1 treated cells (0.68 ±0.09 folds) as shown in figure 4-3D. Furthermore, the phosphorylation of PKC (pan, ser660), which is known to induce p21[9], was abolished by netrin-1 (0.59 ±0.08 folds) (figure 4-3E). In order to further confirm the involvement of PKC in the cell cycle signaling modulated by netrin-1, we pre-treated cells with Gö 6983 for 30 min prior to netrin-1 treatment on cells. As shown in figure 4-3A-E, Gö 6983 pre-treatment abolished the effect of netrin-1. These results suggest PKC lies up-stream of p27, p21, and Rb, facilitating netrin-1-dependent cell cyclying progression in BAECs. Representative images from western blot are shown in figure 4-3A.

Figure 4-3.

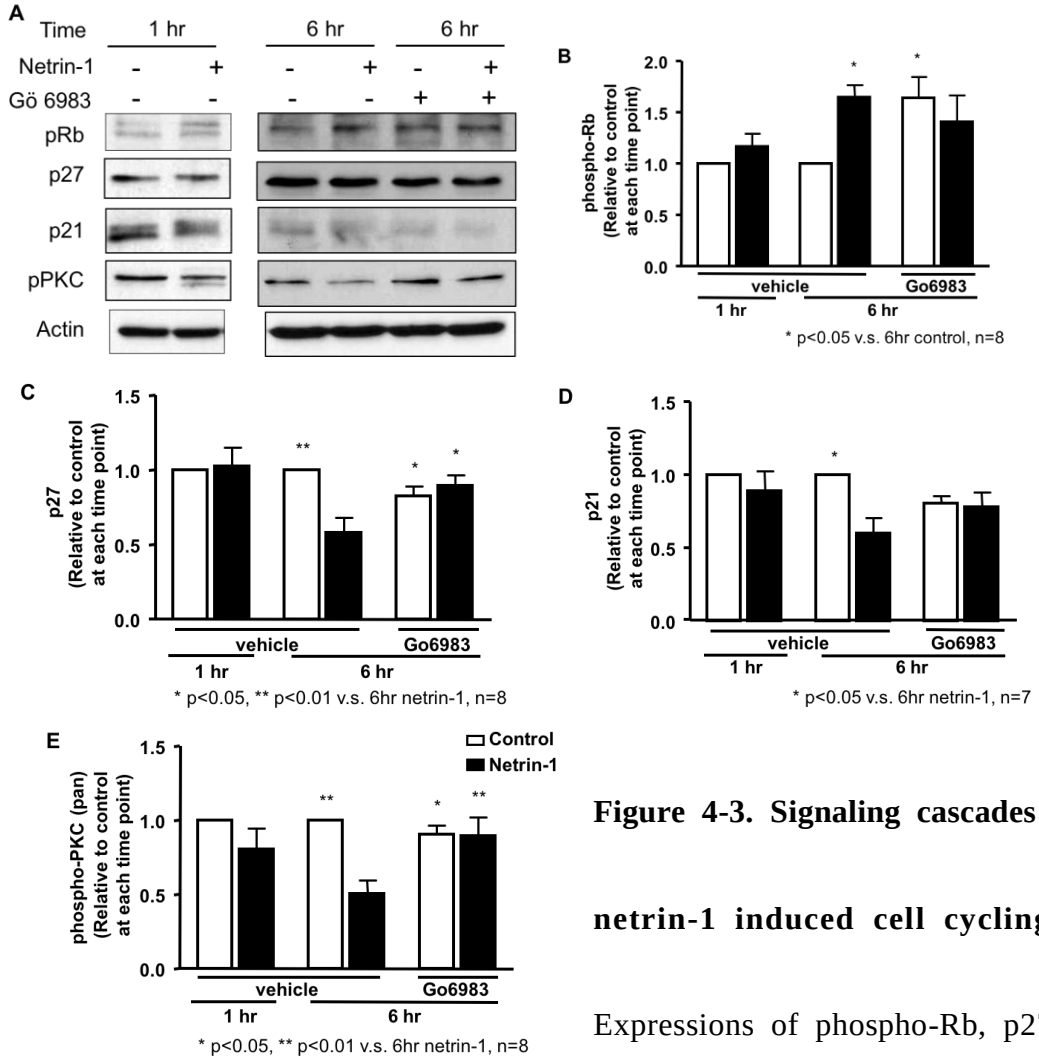

Figure 4-3. Signaling cascades mediating netrin-1 induced cell cycling process. Expressions of phospho-Rb, p27, p21 and phospho-PKC were detected in BAECs with or without pre-treatment of PKC inhibitor Gö 6983 (10nmol/L, 30min) and treatment of netrin-1 for 1hr or 6 hrs. **A)** Representative images of western blot. **B-E)** Quantified data from western blot analysis. P27, p21, and phospho-PKC showed significant decrease by netrin-1 treatment for 6 hrs. While phospho-Rb was increased by netrin-1 at 6 hrs. However, these modifications lead by netrin-1 treatment were abolished by Gö 6983 pre-treatment (n=7-8, *p* values are indicated individually).

4-4. Discussion

In this study, we demonstrated that the promotion of endothelial mitogenic activity stimulated by netrin-1 was at least partially induced by acceleration of cell cycle progression. Moreover, we elucidated, for the first time, that netrin-1 reduces activation of PKC and further decreases p21/p27 that arrest cell cycle from entering S phase, therefore triggering Rb release from E2F complex to contribute to DNA replication. The schematic figure of signaling pathways are shown in figure 4-4.

Netrin-1 exerted modest but statistically significant increase in proliferation and cell cycle progression. The marked effect of netrin-1 shown in wound closure assay [1] might be resulted from synergy of combined responses of migration and proliferation. It also should be noted that these measurements were performed in the absence of any pathological stimuli, so these modest responses might be markedly amplified under pathological conditions.

Consistent with our results, other angiogenic factors such as VEGF activates Rb mediated cell cycle progression via p27 dependent mechanisms. Overexpression of caveolin-1 prevents VEGF-induced down-regulation of the p27 and Rb phosphorylation, and subsequently arrests endothelial cells in the G1 phase[10]. It is known that caveolin-1 inhibits eNOS function; so that the effect of VEGF is similar to netrin-1 in inducing cell proliferation

via eNOS production of NO. Taken together, netrin-1 promoted p27 downregulation and Rb phosphorylation would be downstream event of eNOS activation. At least partially p53 replaces the role of p21 dependent Rb regulation in VEGF stimulated endothelial cells. Our data seem to suggest netrin-1 employs both p27 and p21 to regulate Rb for cell cycle modulation.

We previously showed that there is a feed-forward activating mechanisms between ERK1/2 and eNOS, when netrin-1 binds to DCC in endothelial cells[1]. Moreover, PKC has been greatly recognized as a substrate of ERK1/2 activation [11][12][13], which in turn controls various biological responses including cell proliferation, differentiation, and cell death[14]. On the other hand, PKC also regulates p21 and p27[11], which inhibits the phosphorylation of Rb thus contributing to cell cycle arrest. This outcome of cell mitogen induced by PKC might be the answer of netrin-1's modest effect on proliferation and migration of endothelial cells under a regular condition.

In conclusion, we demonstrated for the first time that the endothelial cell proliferation promoted by netrin-1 is at least partially attributed to the inhibition of cell cycle arrest through activation of Rb,. Moreover, we also identified that PKC lies upstream of p21/p27

regulating Rb activation in the aortic endothelial cells. Further investigations are required to address the physiological significance of PKC and Rb in mediating netrin-1's effects on endothelial cell signaling.

Figure 4-4.

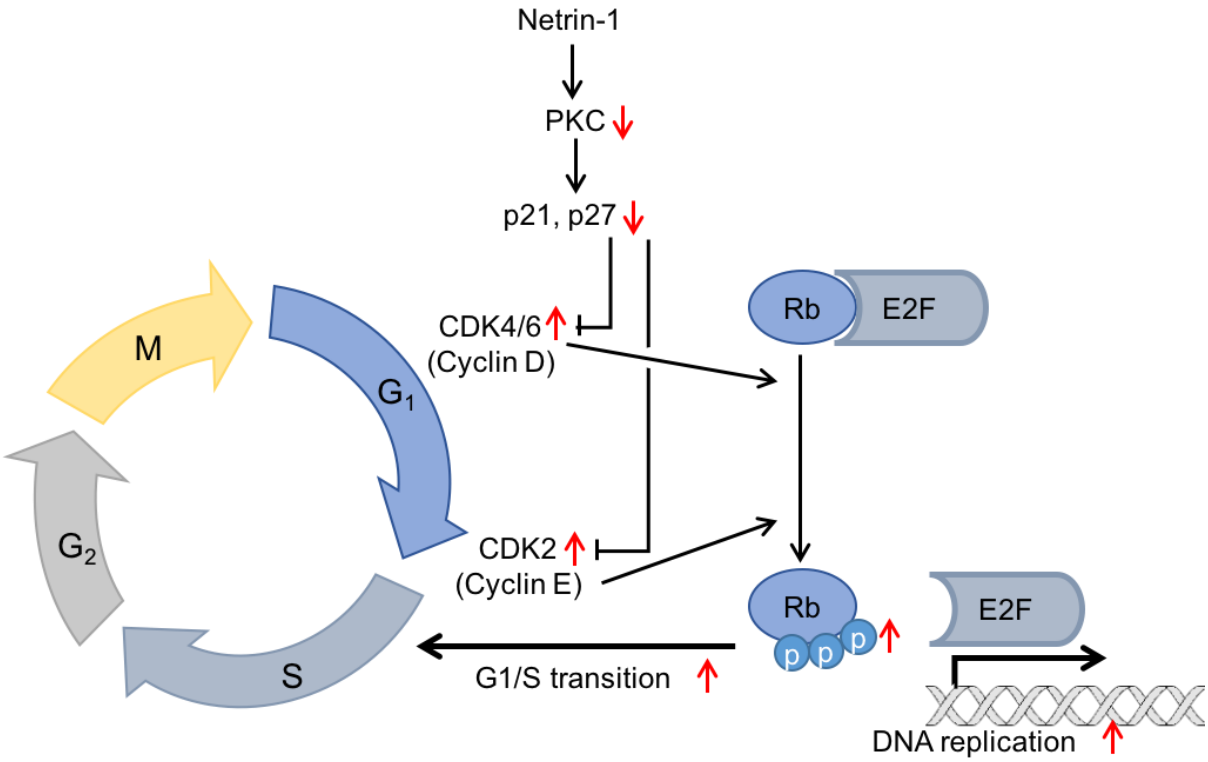

Figure 4-4. Schematic illustration of mechanistic insights underlying netrin-1 induced cell cycling progress. Netrin-1 inhibits PKC activation in BAECs, leading to subsequent inhibition of CDK4/6 inhibitor p21/p27; and this would result in increased Rb phosphorylation to promote cell cycling. Thus netrin-1 accelerates G1/S transition of the cells. Red arrows indicate expression modifications by netrin-1 treatment.

4-5. References

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5. Conclusion

The present study demonstrates a robust vascular protective effect of netrin-1 and netrin-1 preconditioned EPCs using two vascular disease models of femoral artery wire injury for neointimal formation and apoE knockout mice fed high fat diet for atherosclerosis. We have previously discovered that the axon guiding molecular netrin-1 exerts angiogenic effect via DCC receptor dependent production of $NO^{\bullet}$ from endothelial cells. Since $NO^{\bullet}$ bioavailability is the most important factor to maintain vascular homeostasis, we proceeded to examine the potential protective effects of netrin-1 in vascular disease models.

Femoral artery wire injury was employed as a pathological model for neointimal growth following endothelial injury. Since VSMCs are the major component in growing neointima, we created a co-culture system of EC and VSMC to examine netrin-1's effect on VSMC migration and proliferation. The experiments identified a novel signaling pathway of $NO^{\bullet}$/cGMP/p38 MAPK in mediating attenuation of VSMC migration and proliferation. We confirmed elevated levels of bioavailable $NO^{\bullet}$ in injured femoral arteries by electron spin resonance (ESR), indicating that netrin-1 was able to increase $NO^{\bullet}$ production in remaining endothelial cells. Our further data indicate that netrin-1 was able to increase $NO^{\bullet}$ production

in netrin-1 preconditioned EPCs as well, which is required to enhance EPC-dependent revascularization to accelerate vascular repair. The proliferative activity and resistance to oxidative stress induced apoptosis of EPCs are both improved in response to netrin-1 preconditioning. These beneficial effects of netrin-1 treatment are highly significant as the challenges in clinical therapy using EPCs are the sparseness and the vulnerability. In additional experiments, EPCs pre-conditioned with netrin-1 were injected in vivo in the femoral artery wire injury model, where we observed robust vascular protection using much less number of cells at only 500.

In the atherosclerotic model of high fat fed apoE knockout mice, we observed improved phenotypes by netrin-1 infusion, as reflected by reductions in body weight and liver enlargement, improvement in plasma lipid profile, as well as reductions in lesion formation and macrophage infiltration. Monocytes were also examined in this model since they are an indispensable cell type in inflammatory-driven pathology triggered by damaged ECs. We found that netrin-1 inhibited monocyte adhesion to ECs and this effect was abolished in the absence of UNC5B receptor. It suggests that netrin-1 repulsed monocyte adhesion to endothelium via UNC5B and thus decreased macrophage accumulation.

In the last chapter, we examined the cell cycling effect of netrin-1 on ECs, and PKC

and Rb were implicated in the increased proliferation as cell cycling progresses. Although the netrin-1 regulation of PKC/Rb axis was relatively modest, it may however indicate the modest change prevents netrin-1 to have a profound impact on cancer angiogenesis.

Overall, the present study for the first time demonstrates robust vascular protective effects of netrin-1 and netrin-1 pre-conditioned EPCs that are mediated by DCC-dependent increase in $NO^{\bullet}$ production, $NO^{\bullet}$-mediated inhibition of VSMC migration and proliferation, $NO^{\bullet}$-mediated improvements in EPC functions by netrin-1 preconditioning, and UBC5B mediated attenuation of monocyte adhesion to ECs. These data indicate that both netrin-1 and netrin-1 pre-conditioned EPCs may serve as novel therapeutic options for the treatment of vascular diseases.