Leukocyte-specific protein 1 modulates the expression and function of endothelial nitric oxide synthase

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ABSTRACT

Endothelial dysfunction is characteristic feature of many cardiovascular diseases and risk factors such as diabetes, coronary artery disease and hypertension. Recent genome wide association studies have identified polymorphism in human leukocyte-specific protein 1 (LSP1) gene to be associated with development of essential hypertension in humans. Since endothelial dysfunction in hypertension is almost invariably accompanied by alteration in endothelial nitric oxide synthase (eNOS) expression or impairment of NO-dependent vasodilation, we hypothesize that LSP1 has a role in endothelial function via modulation of expression and function of eNOS. We sought to define the role of endothelial LSP1 in regulation of eNOS utilizing in-vitro endothelial cell culture model and LSP1 KO mice. Here we report the novel role of human LSP1 in mediating basal eNOS expression in human macrovascular endothelial cells. Using CRISPR/Cas9 mediated genomic editing to mutate human LSP1 gene and obtain LSP1 deficient endothelial cells, we have demonstrated that transient depletion of LSP1 has induced marked downregulation of eNOS expression, and considerable decrease in nitric oxide synthesis. In addition, LSP1 gain of function via adenovirus mediated overexpression enhances expression of eNOS. We have also revealed using coimmunoprecipitation and confocal microscopy that eNOS and LSP1 associate with each other under basal physiological conditions. Furthermore, LSP1 deficiency in mice induced significant upregulation of eNOS and eNOS uncoupling, associated with enhanced susceptibility of eNOS to proteolytic degradation. Our data clearly propose the novel and the crucial role of endothelial LSP1 in regulation of basal eNOS expression within human endothelial cells and mice cardiovascular tissues.

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DEDICATION

I dedicate my thesis to my parents, my wife, and my newly born twin babies

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

BH2: dihydrobiopterin

BH4: tetrahydrobiopterin

Cas9: CRISPR associated protein

C-terminus: Carboxyl terminus

CMV: Cytomegalovirus

CRISPR: Clustered regularly interspaced short palindromic repeats

DTT: dithiothreitol

DHFR: dihydrofolate reductase

DSBs: double-strand breaks

eNOS: endothelial nitric oxide synthase

EDHF: endothelium derived hyperpolarizing factor

GTP: guanosine-5'-triphosphate

iNOS: inducible nitric oxide synthase

Indels: Insertions deletions

IP: immunoprecipitation

IB: immunoblotting

HCAECs: human Coronary artery endothelial cells

HDR: homology directed repair

HPRT: hypoxanthine guanine phosphoribosyl transferase

HSP90: heat shock protein 90

KO: knockout

Kd: kilodaltons

LSP1: leukocyte-specific protein 1

LT-SDS-PAGE: low temperature sodium dodecyl sulfate polyacrylamide gel electrophoresis

MAPK: mitogen-activated protein kinase

MOI: multiplicity of infection

NO: nitric oxide

NOX: NAPDH oxidase

NOS: nitric oxide synthase

NHEJ: non-homologous end-joining

N-terminus: amino terminus

PAM: protospacer adjacent motif

PGI2: Prostaglandin I2

1. LITERATURE REVIEW

1.1. Endothelial cells biology.

The endothelium is not only a pervasive monolayer of cells lining the interior surface of blood vessels of the vascular tree, acting as a selective barrier to traversing of molecules from vessel lumen to extravascular space, but also it is highly active biological organ (Aird, 2004), serving numerous physiological functions, including adjustment of vasomotor tone, regulation of trafficking of circulating blood elements across the vessel wall, control of vascular permeability, preservation of blood fluidity and therefore ensuring sustained tissue perfusion by inhibition of platelet aggregation and coagulation cascades, and direction of new vessel formation (angiogenesis). Concisely, healthy endothelial cells exhibit vasodilator, antithrombotic and antiadhesive features (Rajendran et al., 2013). The intact endothelium controls basal arterial vascular tone by rapidly sensing the changes in mechanical hemodynamic forces (shear stress and circumferential stretch), and the alterations in the levels of chemical mediators within the blood stream and actively responds with release of several vasoactive substances. Furthermore, the endothelium as an active biological organ can function in endocrine/paracrine fashion and modulate basal tone of underlying vascular smooth muscle (Rajendran et al., 2013), reactivity of the regional blood vessels, and contractility of cardiomyocytes through elaboration of a wide variety of vasoactive factors/autocoids. These substances can generally be divided into relaxative [e.g., nitric oxide (NO), prostacyclin (PGI2), endothelium-derived hyperpolarizing factor (EDHF), and adenosine], and vasoconstrictive [e.g., endothelin-1, thromboxane A2, isoprostanes, superoxide anion, and angiotensin II] substances. Indeed, the regulation of arterial vascular tone is the result of exquisite balance between endothelium dependent relaxation and vasoconstriction responses. Perturbation in the delicate balance of endothelium-derived relaxation and contractile factors is the distinctive feature of essential hypertension. A hallmark of systemic hypertension is the elevation in peripheral vascular resistance resulting from augmented vasoconstriction and/or attenuated endothelium dependent vasodilation (De Artinano & Gonzalez, 1999).

1.2. Nitric oxide signalling and nitric oxide synthases

Nitric oxide is a short-lived (half life of a few seconds), locally acting gaseous biomolecule and is synthesized from two substrates (L-arginine and molecular oxygen) by one of the three distinctive nitric oxide synthase (NOS) isoforms, endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) nitric oxide synthases. Shear stress is a significant physiological stimulus (Chiu & Chien, 2011) for nitric oxide release, and nitric oxide production is significantly higher in macrovascular arterial endothelial cells compared to microvascular endothelial cells (Geiger, Stone, Mason, Oldham & Guice, 1997). Furthermore, throughout the arterial vascular bed, the expression of eNOS (Chiu & Chien, 2011) is more robust in high shear-stress regions of large arteries compared to regions exposed to low shear stress forces. The expression of eNOS is also greater in endocardial endothelium (Aird, 2007) in contrast to myocardial microvascular endothelial cells. The expression of eNOS is low in smaller arteries, and is not expressed in capillary endothelial cells (Heiss, Rodriguez-Mateos & Kelm, 2015). Endothelial cells signal shear stress *via* potassium channels and cytoskeleton (Govers & Rabelink, 2001).

Shear stress and multiple receptor agonists (bradykinin, acetylcholine, histamine, thrombin, ADP/ATP) can trigger eNOS activation via surge in intracellular calcium concentration (Hennenberg, Trebicka, Sauerbruch & Heller, 2008). Shear stress (Govers & Rabelink, 2001) can also elicit eNOS activity by induction of protein kinase B (Akt kinase) and subsequently eNOS serine phosphorylation. Vascular endothelial growth factor (VEGF) mimics shear stress and induces eNOS activity in both phosphorylation and calcium dependent patterns (Govers & Rabelink, 2001).

Nitric oxide synthase (NOS) is a homodimer enzyme consisting of two identical monomers (Andrew & Mayer, 1999; Campbell, Smith, Potter, Carragher & Marletta, 2014). For the enzyme to be catalytically active and generate nitric oxide, NOS must attach cofactors and dimerize. Each NOS monomer protein has N-terminal oxygenase and C-terminal reductase domains connected by calmodulin linker region which binds calcium in eNOS and nNOS isoforms (Campbell, Smith, Potter, Carragher & Marletta, 2014). The activities of both eNOS and nNOS are calcium dependent, whereas, iNOS bind calmodulin completely independent of changes in intracellular

calcium concentration (Spratt, Taiakina, Palmer & Guillemette, 2007). Oxygenase domain has active heme center of NOS enzyme which catalyzes the conversion of amino acid L-arginine to nitric oxide and non-proteinogenic amino acid L-citrulline (Du, Yeh, Berka, Wang & Tsai, 2003). The oxygenase requires essential cofactor tetrahydrobiopterin (BH4) and heme prosthetic group. The reductase domain binds three cofactors (NADPH, FAD, FMN) and function to supply reducing equivalents to heme center of oxygenase domain (Du, Yeh, Berka, Wang & Tsai, 2003). Zinc ions coordinate the stabilization of the dimer and are positioned at the interface of two oxygenase domains (Raman, Li, Martasek, Kral, Masters & Poulos, 1998). It is important to emphasize that BH4 is the most crucial cofactor for NOS activity (Crabtree et al., 2009). Under physiological conditions, the availability of BH4 as critical cofactor for eNOS activity is determined by two pathways, 1) new synthesis from GTP, the rate limiting step in this pathway is catalyzed by GTP cyclohydrolase, and 2) recycling of dihydrobiopterin (BH2, product of BH4 as result of oxidative stress) to BH4 by dihydrofolate reductase (Crabtree, Hale & Channon, 2011).

Normally, NOS activity is "coupled" implying that the oxidation of L-arginine is coupled to reduction of molecular oxygen. However, when there is diminished bioavailability of BH4, the reduction of molecular oxygen is not coupled to oxidation of L-arginine (NOS uncoupling) and NOS functions analogously to NADPH oxidase (NOX) and generation of superoxide anions (instead of nitric oxide) will ensue. Several potential mechanisms have been proposed to account for eNOS uncoupling and diminished nitric oxide production, including limited availability of BH4, deficiency of substrate L-arginine (Crabtree, Hale & Channon, 2011), increased inactivation of nitric oxide by reactive oxygen species (ROS), upregulation of arginase activity or expression, increase in the levels of naturally occurring arginine analogues, ADMA (asymmetric dimethylarginine) and higher ratio of oxidized to reduced (GSSG/GSH) glutathione. Endothelial NOS and neuronal NOS are the two constitutively active isoenzymes whereas inducible NOS is expressed in circulating monocytes, macrophages and neutrophils under the conditions of inflammation, immune response, and septicemia.

Nitric oxide is the most potent endogenous vasodilator and exerts numerous vasoprotective and cardioprotective effects within the circulatory system (Davignon & Ganz, 2004). Adequately produced nitric oxide is universally considered as the marker of healthy circulation. Nitric oxide synthesized by eNOS is a vital mediator of endothelium dependent relaxation, maintain normal

coronary blood flow, inhibit the proliferation of vascular smooth muscle, and counteracts the actions of endothelium-derived vasoconstrictors. Moreover, nitric oxide inhibits leukocyte-endothelial interactions by suppressing the expression of integrins and other adhesion molecules on the surface of endothelial cells. Nitric oxide also acts as anti-thrombotic by inhibition of platelet activation and aggregation and by the fine modulation of balance between pro-thrombotic and anti-thrombotic stimuli. In addition, nitric oxide possesses anti-oxidant properties, inhibits oxidation of low-density lipoproteins (LDLs) and exerts anti-atherogenic effect preventing development of atherosclerosis. Most of the beneficial effects (vasodilator, anti-inflammatory, anti-thrombotic) are evident at low physiological concentrations of nitric oxide formed by the two constitutive NOS isoforms- eNOS and nNOS. On the other hand, much higher cytotoxic concentrations of nitric oxide generated by iNOS activity ordinarily have more deleterious ramifications on vascular homeostasis. The expression of iNOS is usually induced by pro-inflammatory cytokines and infectious pathogens, for example, TNF-alpha (TNF α), interleukin-1β (IL- 1β), bacterial lipopolysaccharide (LPS), and gamma interferon (γ IFN). Furthermore, iNOS-derived NO participate in the killing of microbes and host innate immune responses. There is growing body of evidence that not only link upregulation of iNOS activity to pathogenesis of hypertension, but also suggests that there is an inflammatory component (Virdis et al., 2011) in hypertensive states. J. Smith (Smith, Santhanam, Bruning, Stanhewicz, Berkowitz & Holowatz, 2011) and colleagues have reported the increased expression of iNOS in microvasculature of hypertensive patients is associated with microvascular dysfunction and impaired NO-dependent relaxation of cutaneous arterioles. They have has demonstrated that inhibition of iNOS activity results in restoration of NO-dependent vasodilatation Furthermore, sustained and high-output nitric oxide release by iNOS usually yields large quantities of superoxide and peroxynitrite radicals in redox tissue milieu that is peculiar of hypertension. Superoxide quenches nitric oxide (thus deactivating nitric oxide), resulting in the formation of peroxynitrite (Pacher, Beckman & Liaudet, 2007) and vasoconstriction. Peroxynitrite as one of most oxidant and detrimental reactive nitrogen species promotes vasoconstriction and hypertensive phenotype via diverse mechanisms which are briefly discussed below. Apart from the contribution by iNOS -derived superoxide and peroxynitrite to hypertension, iNOS also activates arginase and promote eNOS uncoupling via competition for the substrate arginine between eNOS and arginase, therefore, further impair endothelium dependent vasodilatation.

There are three major scavenging enzymes (superoxide dismutase, glutathione peroxidase, and catalase) that can neutralize detrimental effects of reactive oxygen species. Among which, copper magnesium-SOD is of paramount significance. In fact, SOD quenches superoxide before it can react with NO to form peroxynitrite, prolonging the half-life of residual nitric oxide, and therefore enhancing vascular function. Apart from eNOS uncoupling mediated production of superoxide, xanthine oxidase, NADPH oxidase (NOX), and mitochondrial oxidative phosphorylation enzymes also contribute to superoxide anion production (Wolin, Gupte & Oeckler, 2002) in the vascular system. It has been reported that xanthine oxidase could be the largest source of superoxide radical oxygen species in the vascular system (Miyamoto, Akaike, Yoshida, Goto, Horie & Maeda, 1996). Superoxide free radicals produced by eNOS uncoupling or xanthine oxidase interact with nitric oxide forming peroxynitrite (Jourd'heuil, Jourd'heuil, Kutchukian, Musah, Wink & Grisham, 2001; Sawa, Akaike & Maeda, 2000), very reactive extremely unstable detrimental anion that readily oxidizes BH4 (Chen et al., 2010), causing further depletion of BH4 and leading to vicious cycle of vascular oxidative stress.

Under certain pathological situations in which there is a high degree of oxidative stress or chronic inflammation, peroxynitrite reacts with and nitrates specific tyrosine moieties on some candidate proteins, giving rise to the formation of 3-nitrotyrosine modified proteins. This selective pathological process is designated as protein tyrosine nitration (Daiber & Munzel, 2012). Protein tyrosine nitration reflects posttranslational modification (Radi, 2013) mediated by nitric oxide-derived reactive nitrogen species (RNS) and is known to influence the function of numerous proteins within the physiological systems. 3-Nitrotyrosine modified proteins have the ability to trigger the activation of immune system leading to the generation of specific antibodies against nitrated proteins (Daiber & Munzel, 2012). Tyrosine nitration together with specific immune responses against nitrotyrosine proteins have been implicated in a variety of chronic inflammatory, autoimmune, and cardiovascular diseases, such as rheumatoid arthritis, systemic lupus erythematosus, and atherosclerosis. One of the particularly unique reactions catalyzed by peroxynitrite is tyrosine nitration and inactivation of prostacyclin synthase (PGIS) (Zou, 2007) which consequently results in reduction in levels of beneficial prostacyclin (PGI2), and favors the buildup of non-metabolized prostaglandin endoperoxide H2 (PGH2), therefore leading to enhanced synthesis of deleterious thromboxane A2. This in turn will cause further impairment of the endothelium-dependent vasorelaxation and will have a major impact on the cardiovascular

function not only by shifting the balance from vasodilator PGI2 to vasoconstrictor PGH2/TXA2, but also from attenuating anti-inflammatory, anti-thrombotic, and vasoprotective actions of PGI2, and promoting prothrombotic, pro-adhesive, pro-atherogenic and vascular remodeling effects of PGH2/TXA2 (Daiber & Munzel, 2012; Zou, 2007). Peroxynitrite mediated PGIS tyrosine nitration has been reported in multiple cardiovascular diseases, such as hypertension, diabetes, atherosclerosis, and ischemia-hyperperfusion injury (Zou, 2007). Furthermore, peroxynitrite triggers peroxidation of lipids in the cellular membrane, an oxidative self-propagating process, culminating in production of other vasoconstrictor compounds, for example, F2-isoprostanes (8-iso-prostaglandin F2 alpha). F2-isoprostanes are powerful renal vasoconstrictors and potentiate (Sametz, Grobuschek, Hammer-Kogler, Juan & Wintersteiger, 1999) the effects of angiotensin 2, noradrenaline and endothelin, thus, eliciting a profound rise in peripheral vascular resistance and systemic blood pressure. The detection of 3-nitrotyrosine (Cuzzocrea, Mazzon, Dugo, Di Paola, Caputi & Salvemini, 2004) by immunohistochemical or proteomic methods within the tissues is broadly utilized as an assay of endogenous peroxynitrite activity and as a marker of in vivo nitrosative stress. Apart from the contribution by peroxynitrite formation, protein tyrosine nitration can also be mediated by myeloperoxidase pathway (Kettle, van Dalen & Winterbourn, 1997). Myeloperoxidase (Hataishi, Kobayashi, Takahashi, Hirano, Zapol & Jones, 2002) induces the generation of hypochlorite (HOCl), which in turn interacts with nitrite (NO_2) to produce NO_2Cl, a potent nitrating and chlorinating substance. The morphological identification of chlorotyrosine along with nitrotyrosine within the tissues by immunohistochemistry (Hataishi, Kobayashi, Takahashi, Hirano, Zapol & Jones, 2002) is a fundamental criterion to differentiate between myeloperoxidase and peroxynitrite pathways.

1.3. Intracellular regulation of eNOS

Apart from coupling status of eNOS, eNOS is subject to complex phases of regulation in cardiovascular physiology (Figures 1,1 and 1.2).

1.3.1. Transcriptional regulation of eNOS.

Shear stress forces contribute largely to tissue specific expression of eNOS, and the expression of eNOS is primarily restricted to endothelium of large to medium sized arterial blood vessels. It has been revealed that shear stress increase eNOS mRNA abundance by transcriptional mechanism involving activation of eNOS promoter via existence of several shear stress response elements (Ziegler, Silacci, Harrison & Hayoz, 1998). In addition, shear stress has been demonstrated to stimulate transcription of eNOS through activation of tyrosine kinase c-Src signalling pathway (Davis, Cai, Drummond & Harrison, 2001). Shear stress may also induce transcription of eNOS through transient activation of NFκB (Davis, Grumbach, Fukai, Cutchins & Harrison, 2004). Beside regulation by shear stress forces, eNOS promoter harbors binding sites for Sp1 and GATA2 transcription factors which are essential for basal transcription of eNOS gene (Zhang, Min & Sessa, 1995). Hormones such as Estrogen (Chambliss & Shaul, 2002), and insulin (Kuboki et al., 2000) upregulate expression of eNOS at transcriptional level.
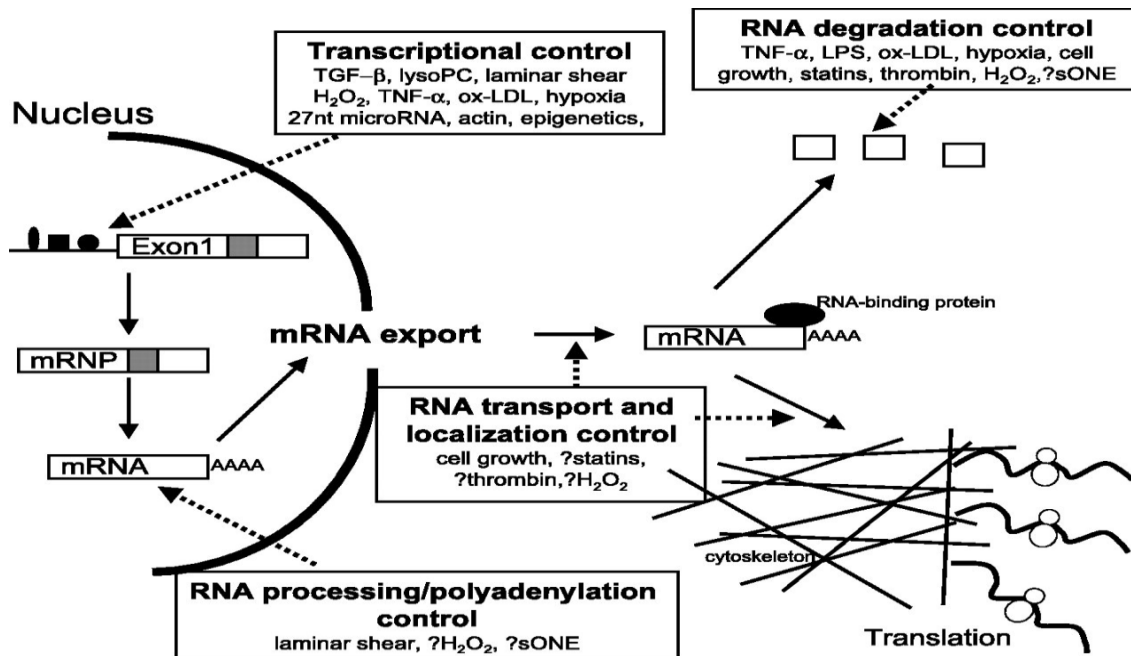
Figure 1.1: Transcriptional and post-transcriptional regulation of eNOS

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7

On the other hand, several other factors have been identified to decrease transcriptional expression of eNOS. Among which, NO itself exerts negative feedback regulation of eNOS via cGMP-dependent downregulation process of eNOS gene expression (Vaziri & Wang, 1999). In addition, another determinant of eNOS gene expression is tumor necrosis factor-alpha (TNF- α) which has been shown to inhibit promoter activity of eNOS gene (Anderson, Rahmutula & Gardner, 2004). Furthermore, oxidized low density lipoproteins (LDL) (Ma et al., 2006), and hypoxia (McQuillan, Leung, Marsden, Kostyk & Kourembanas, 1994) are among other factors that have been identified to lower gene expression of eNOS.

1.3.2. Post-transcriptional regulation of eNOS.

An important aspect of regulation of eNOS at post-transcriptional level is the stability of eNOS mRNA transcripts. eNOS mRNA abundance reflects the balance between eNOS transcriptional expression and eNOS mRNA degradation. Among factors that influence the stability of eNOS mRNA, Shear stress increases eNOS mRNA levels via post-transcriptional mechanism (Ziegler, Silacci, Harrison & Hayoz, 1998). In addition, VEGF and hydrogen peroxide increases stability of eNOS mRNA (Bouloumie, Schini-Kerth & Busse, 1999; Drummond, Cai, Davis, Ramasamy & Harrison, 2000). TNF-alpha, lipopolysaccharides (endotoxins), oxidized LDL particles, thrombin, and hypoxia on the other hand reduce stability of eNOS mRNA transcripts (Searles, 2006).

1.3.3. Post-translational regulation of eNOS.

1.3.3.1. Subcellular localization of eNOS.

Localization of eNOS within subcellular compartments is viewed as an important locus of regulation of eNOS activity. eNOS resides in plasma membrane within specific membrane invaginated structures known as caveolae. Plasma membrane of endothelial cells is enriched with caveolae, and within caveolae, caveolin-1 protein directly interacts with eNOS and inhibits its activity (Mineo & Shaul, 2012). Caveolin-1 mediated inhibition of eNOS activity occurs partially through occupation of calmodulin binding site. Targeting of eNOS to caveolae relies on specific acylation type post-translational modifications known as myristoylation and palmitoylation

8

(Shaul et al., 1996). eNOS localization to caveolae enables the enzyme to interact with other proteins such as bradykinin receptor and cationic amino acid transporter CAT-1 (transporter for arginine NO substrate). Since estrogen, VEGF, and G protein coupled receptors, calcium pump modulators, and protein kinase C are also localized in Caveolae, it appears that eNOS concentrated in caveolae renders the enzyme in close proximity to other key signalling molecules that are crucial to modulation of eNOS activity (Govers & Rabelink, 2001; Shaul et al., 1996).
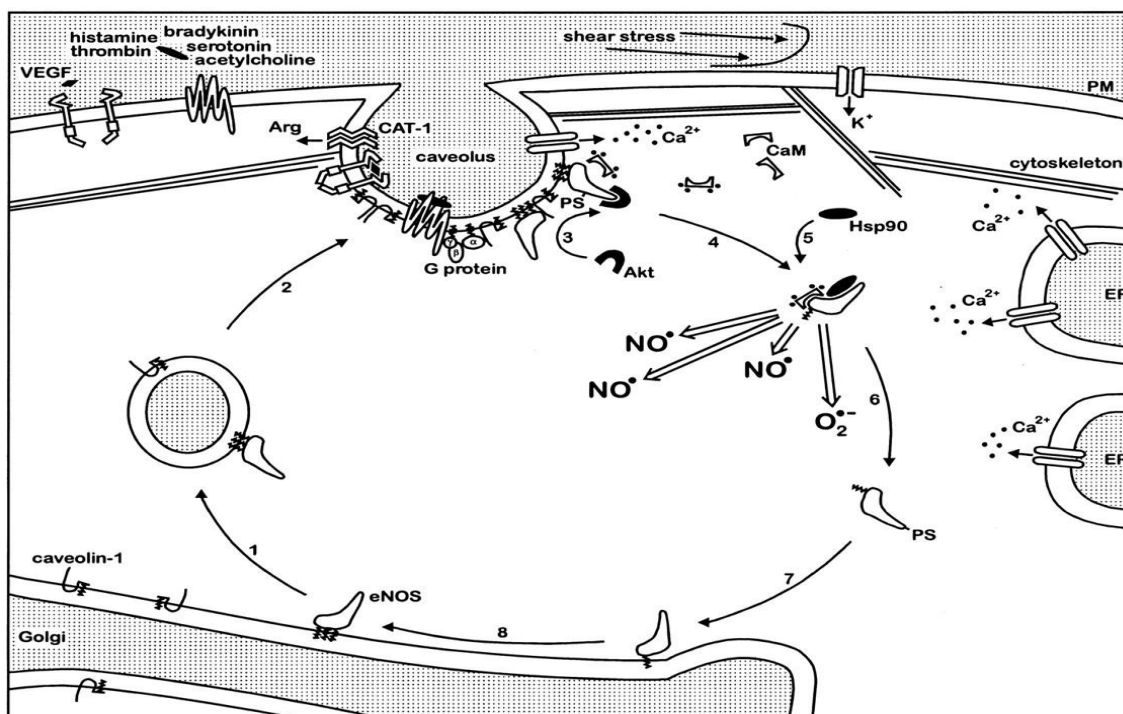
Figure 1.2: Post-translational activation and deactivation of eNOS

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1.3.3.2. Calcium/Calmodulin regulation of eNOS

Calmodulin is vital regulator of eNOS activity. Calmodulin-dependent activation of eNOS is largely influenced by intracellular calcium levels (Busse & Mulsch, 1990). Shear stress, and calcium-mobilizing stimuli such as bradykinin, acetylcholine, calcium ionophore, and estradiol trigger eNOS activation via increase in intracellular calcium. Elevation in intracellular calcium levels enhances binding of calmodulin to eNOS. Calmodulin binding facilitates transfer of electrons from NADPH in reductase domain to heme in oxygenase domains of eNOS, disrupt inhibitory eNOS-caveolin-1 interaction, and maximizes catalytic activity of the enzyme

9

(Dudzinski & Michel, 2007). However, at suboptimal intracellular calcium concentrations, auto-inhibitory loop in reductase domain destabilize calmodulin binding, which in turn disrupt flow of electrons from reductase to oxygenase domains and inhibit catalytic activity of eNOS (Dudzinski, Igarashi, Greif & Michel, 2006; Forstermann & Sessa, 2012).

1.3.3.3 Phosphorylation of eNOS

Apart from subcellular localization and protein-protein interactions mediated regulation, eNOS is subject to post-translational dynamic regulation by phosphorylation at multiple specific consensus serine and threonine amino acid residues (Mount, Kemp & Power, 2007). Phosphorylation of serine (1177) residue near carboxyl-terminus by Akt, AMPK, caMK-2, and PKA activates eNOS, whereas phosphorylation of threonine (495) residue by PKC inhibits enzyme activity by impeding calmodulin binding to calmodulin-linker region (Kukreja & Xi, 2007; Mount, Kemp & Power, 2007). Despite being critical positive phosphorylation site for activation of eNOS and synthesis of NO, phosphorylation of serine (1177) by Akt could also lead to increased superoxide generation from eNOS at low calcium concentration (Chen, Druhan, Varadharaj, Chen & Zweier, 2008). Several other regulatory serine phosphorylation sites in eNOS have been revealed (Bauer et al., 2003) with different effects on eNOS protein-protein interactions, basal and agonist stimulated NO production. Among multiple serine phosphorylation sites, serine (635) and serine (116) represent additional crucial positive and negative regulatory sites, respectively.

1.4. Endothelial dysfunction and role of eNOS in hypertension.

Endothelial dysfunction is a pathological concept frequently utilized to imply diminished nitric oxide bioavailability manifested as the impairment of the endothelium-mediated vasorelaxation (Davignon & Ganz, 2004; De Artinano & Gonzalez, 1999). Almost all major cardiovascular conditions (hypertension, diabetes, stroke, atherosclerosis, angina pectoris and

coronary artery disease) are associated to some extent with endothelial dysfunction or impaired endothelium dependent vasodilation. Endothelial dysfunction as manifested by reduced availability of NO is common phenomenon in hypertension and is consequence of decreased NO synthesis by eNOS or augmented NO inactivation (Touyz & Schiffrin, 2001; Zalba et al., 2001). Patients with essential hypertension have reduced reserve of endothelium response function (Schmieder et al., 1997). Alterations of eNOS gene expression or activity has been demonstrated to be involved in hypertension. eNOS knockout mice are hypertensive (Huang et al., 1995). Inhibition of eNOS activity with L- arginine analogues results in sharp elevation of mean arterial blood pressure (Rees, Palmer & Moncada, 1989). Transgenic mice overexpressing eNOS of bovine origin have lowered blood pressure (Ohashi et al., 1998). Although endothelial dysfunction is characterized by reduced output of bioactive nitric oxide, it is not necessarily caused by lowered expression of eNOS. In fact, several models of hypertension are associated with upregulation, rather than downregulation of eNOS expression (Li, Wallerath, Munzel & Forstermann, 2002). The upregulation of eNOS expression in the setting of endothelial dysfunction could be attributed to the loss of negative feedback loop normally exerted by nitric oxide (Vaziri & Wang, 1999), or induction of eNOS expression by reactive oxygen species, such as hydrogen peroxide (Drummond, Cai, Davis, Ramasamy & Harrison, 2000; Zhen, Lu, Wang, Vaziri & Zhou, 2008) or combination of both factors (Berggard, Linse & James, 2007). Mechanistically, NO inactivates eNOS by S-nitrosylation (Ravi, Brennan, Levic, Ross & Black, 2004) which is distinguished posttranslational modification mediated by NO and represent cGMP-independent signalling pathway of NO (Ahern, Klyachko & Jackson, 2002). Nitric oxide availability as vasodilator often depends on the equilibrium between NO production by NOS isoforms and nitric oxide scavenging by superoxide free radicals or hemoglobin. However, since eNOS expression is preferentially confined to (Fish et al., 2005) endothelium of medium-to-large sized conduit arteries (e.g. Aorta). Therefore, the vasodilator action of nitric oxide is less prominent in small resistance arteries such as mesenteric arteries, where NO independent relaxation factors (Endothelium derived hyperpolarizing facto) play a more crucial role

1.5. EDHF (endothelium derived hyperpolarizing factor)

EDHF is a broad concept with several candidates have been suggested, including (Larsen, Zhang & Gutterman, 2007) epoxyeicosatetraenoic acids (EETs, product of arachidonic acid metabolism via cytochrome P450 monoxygenase), efflux of potassium ions to extracellular space, myoendothelial gap junctions and C-type natriuretic peptide. Potassium channels (Jackson, 2006) contribute significantly to the endothelium hyperpolarization and play a pivotal role in vasodilatation of small resistance vessels, such as renal, mesenteric and coronary arteries. In fact, the rapid rise in intracellular calcium within arterial endothelial cells triggered by receptor agonists (acetylcholine, bradykinin, ATP, substance P) and shear stress evokes opening of small (sKCa) and intermediate (IKCa) conductance calcium activated potassium channels, leading to efflux of potassium ions to extracellular space and hyperpolarization of the endothelial cell membrane. Furthermore, this alteration in the membrane potential of the endothelial cells, or the escalation in shear stress forces may initiate the activation of inwardly rectifying (KIR) potassium channels, promoting further change in membrane potential, and amplification of endothelial cell hyperpolarization response. Endothelial hyperpolarization (Jackson, 2006) generated by IKCa and sKCa potassium channels in small resistance arteries spreads to the underlying vascular smooth cells through myoendothelial gap junctions, provoking closure of voltage gated calcium channels (VGCC) on the smooth muscle cell membrane, causing decrease in intracellular calcium, and leading to vasodilation. Endothelium and smooth muscle cell hyperpolarization can potentially be transmitted bidirectionally along the path of resistance arteries via gap junctions electrically linking juxtaposing arterial endothelial and smooth muscle cells, resulting in spread of hyperpolarization wave and more regionally augmented vasodilatation.

1.6. LSP1 (leukocyte-specific protein 1)

LSP1 is F-actin binding and cytoskeleton associated protein (Jongstra-Bilen, Janmey, Hartwig, Galea & Jongstra, 1992) and putative calcium-binding protein (Klein, Jongstra-Bilen, Ogryzlo, Chong & Jongstra, 1989), that initially has been detected in lymphocytes, and cells of hematopoietic origin, such as, neutrophils, macrophages, monocytes, Langerhans cells (Pulford, Jones, Banham, Haralambieva & Mason, 1999). Because of its original identification in Lymphocyte, LSP1 has been named lymphocyte specific protein-1. LSP1 has recently been identified in endothelial cells (Liu et al., 2005). Furthermore, LSP1 expression was also reported in fibrocytes and hepatocytes. Besides normal distribution of LSP1 in healthy cells, abnormal pattern of LSP1 expression and single nucleotide polymorphism in LSP1 gene have been observed in variety of neoplasms, for instance, hepatocellular carcinoma (Zhang et al., 2016), breast carcinoma (Chen, Qi, Qiu & Zhao, 2015), and vast array of leukemias (Miyoshi, Stewart, Kincade, Lee, Thompson & Wall, 2001) and lymphomas (Marafioti, Jabri, Pulford, Brousset, Mason & Delsol, 2003) of B-cell origin.

The correlation between LSP1 expression or function and neoplastic transformation suggests the significant role of LSP1 in signal transduction and further support the concept that LSP1 has yet unknown mechanism of actions. LSP1 is involved in regulation of leukocyte motility (Jongstra-Bilen et al., 2000) and trans-endothelial migration (Liu et al., 2005), phagocytosis, production of reactive oxygen species (ROS) by neutrophils, histamine induced Endothelial hyperpermeability (Liu et al., 2005), remodeling of actin cytoskeleton (Howard, Hartwig & Cunningham, 1998; Li, Zhang, Aaron, Hilliard & Howard, 2000), adhesion of neutrophils to extracellular matrix protein fibrinogen (Wang et al., 2002), skin wound healing and fibrosis (Wang et al., 2007), and anti-IgM mediated apoptosis of B lymphocytes (Jongstra-Bilen, Wielowieyski, Misener & Jongstra, 1999). Moreover, genome wide scale studies have identified single nucleotide polymorphisms (SNPs) in LSP1 gene to be significantly replicated in patients with primary hypertension (Azam & Azizan, 2018; Johnson et al., 2011). LSP1 has also been shown to regulate migration of T-lymphocytes in patients with rheumatoid arthritis (Hwang et al., 2015). LSP1 is overexpressed in neutrophil actin dysfunction syndrome, rare actin immunodeficiency genetic disorder characterized by impaired neutrophil motility and increased susceptibility to recurrent bacterial infections. Mouse and human LSP1 are composed of 330 and 339 amino acid residues in length, respectively (Jongstra-Bilen &

Jongstra, 2006). The estimated molecular weight of Human LSP1 (Carballo, Colomer, Vives-Corrons, Blackshear & Gil, 1996; Huang, Zhan, Ai & Jongstra, 1997; Pulford, Jones, Banham, Haralambieva & Mason, 1999) is 60kD. Interestingly, earlier study has demonstrated that human LSP1 is not single entity (Carballo, Colomer, Vives-Corrons, Blackshear & Gil, 1996), but rather it is two proteins designated as P55/P60, implying human LSP1 with apparent molecular weight of 55 or 60 kD, respectively. Furthermore, the shift in molecular weight from 55 to 60 kD represent difference in phosphorylation status of human LSP1. P60 LSP1 was most predominant PKC substrate in human neutrophils. The possibility of other isoforms of human LSP1 is further supported by southern blot analysis of human kidney genomic DNA which show pattern of multiple bands mostly corresponding to majority of coding region of human LSP1 in contrast to simple pattern observed after southern blotting analysis of mouse genomic DNA (Jongstra-Bilen, Young, Chong & Jongstra, 1990). Human LSP1 and mouse LSP1 shares considerable amino acid sequence similarity, particularly over C-terminus (Jongstra-Bilen, Young, Chong & Jongstra, 1990). However, this homology is not uniformly distributed over entire LSP1 molecule. The acidic N-terminal half of LSP1 has putative calcium binding motif, while the basic C-terminal half of LSP1 is characterized by presence of F-acting binding domains. Murine LSP1 has two possible calcium binding sites, whereas human LSP1 has one putative calcium binding site. The sequences of calcium binding motifs are different between human and mouse LSP1, implying functional rather than strict sequence conservation (Jongstra-Bilen, Young, Chong & Jongstra, 1990). Mouse LSP1 in granulocytes is expressed in three various cellular compartments (60% in cytosol, 25% in cytoplasmic side of plasma membrane and 15% in cytoskeleton) (Jongstra-Bilen, Janmey, Hartwig, Galea & Jongstra, 1992). In contrast to leukocyte LSP1 which is mostly expressed in cytosol, mouse endothelial LSP1 is localized mainly in the nucleus with a small fraction associated with actin cytoskeleton (Liu et al., 2005). The carboxyl terminal domains of mouse and human LSP1 contain several conserved serine and threonine residues that serve as potential phosphorylation sites by MAPK activated protein kinase2 (MK2) or protein kinase C (PKC) (Jongstra-Bilen & Jongstra, 2006). LSP1 promoter has been shown to harbor binding motifs for Ets, SP1, and C/EBP transcription factors which control basal activity of promoter (Omori, Smale, O'Shea-Greenfield & Wall, 1997). LSP1 promoter is also controlled by presence of *cis*-acting silencer and anti-silencer elements (Malone, Omori, Gangadharan & Wall, 2001).

LSP1 KO mice were generated from WT (SVJ129) mice by specific targeting of exon 1 of LSP1 gene which is critical for transcriptional expression of LSP1 (Jongstra-Bilen et al., 2000). LSP1-knockout mice showed increased transendothelial migration of leukocytes to inflamed tissues compared to WT mice, suggesting that LSP1 has negative role in leukocyte transendothelial migration (Jongstra-Bilen et al., 2000). LSP1 KO mice have also accelerated wound healing which is consistent with increased infiltration of leukocytes and fibrocytes (Wang et al., 2007). In addition, LSP1 knockout mice exhibited increased production of superoxide radicals in neutrophils (Hannigan, Zhan, Ai & Huang, 2001), further supporting crucial role of LSP1 in inflammation. Study on the role of LSP1 in endothelial permeability has demonstrated that LSP1 KO mice have diminished response to histamine induced microvascular permeability (Liu et al., 2005), suggesting that endothelial LSP1, particularly cytoskeleton associated LSP1 might play positive role in contraction of endothelial cells.. Silencing and overexpression of LSP1 in murine microvascular SVEC4-10EE2 endothelial cell has revealed the novel role of endothelial LSP1 in regulation of GATA2 transcription factor and PECAM1 adhesion molecule expression (Hossain et al., 2015). Furthermore, it appears that endothelial LSP1 in the nucleus has significant role in transcription, since silencing of LSP1 downregulates GATA2 and PECAM1, and overexpression of LSP1 enhances expression of GATA2 and PECAM1. However, there was no statistically significant difference in expression of PECAM1 in LSP1 KO compared to WT mice.

Collectively, previous studies suggest that LSP1 is expressed in multiple tissues and numerous cell types and is implicated in multitude of physiological and pathological processes in the human body, including inflammation, host defenses, autoimmunity, and malignancy. Taken together, it seems that LSP1 is a very significant signalling molecule. While role of LSP1 in inflammatory responses has been extensively studied, there is scientific gap in understanding function of endothelial LSP1, why it is preferentially localized to nucleus, and how it dictates endothelial function, particularly whether endothelial LSP1 is involved in regulation of eNOS expression and endothelial-dependent vasodilatation.

1.7. CRISPR-Cas9 mediated genome editing.

Genome editing is a novel process of making specific changes in DNA sequence in order to permanently modify genome of living cells or organisms. Genome editing has been accomplished by use of engineered endonucleases that are designed to cut DNA at precise locations (Hsu, Lander & Zhang, 2014). The outcome of genome editing largely depends on naturally occurring cellular DNA repair mechanisms. Double strand breaks (DSB) induced by programmable endonucleases activate endogenous DNA repair pathways, either error prone non-homology end joining (NHEJ) or template dependent error free homology directed repair. Non-homologous end joining is commonly used to generate gene knockout. Multiple engineered nuclease systems have been utilized for genome editing. Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) although they were the first invented programmable nuclease tools, they rely primarily on protein guided recognition of target DNA (Hsu, Lander & Zhang, 2014), which in addition to their large size make them difficult to design, and would complicate delivery process and targeting of new genes because it is challenging to customize protein component of system for each new gene target (Gupta & Musunuru, 2014). CRISPR/Cas9 system was initially discovered in prokaryotes (bacteria and archaea) as an adaptive immune response against (Barrangou et al., 2007) foreign invaders (Bacteriophages). CRISPR/Cas9 stands for **C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats CRISPR-**A**ssociated **P**roteins **9.** Distinct from ZFN and TALENS, CRISRP/Cas9 system depends on RNA directed recognition of DNA sequence (Chandrasegaran & Carroll, 2016) which make it simpler to engineer and deliver, and easier to recruit cas9 endonuclease to any gene of interest. Furthermore, CRISPR/Cas9 can be readily adapted to any target gene of interest just by changing 20bp sequence of guide RNA (Gupta & Musunuru, 2014). Another advantage of CRISPR/Cas is its multiplexity which allow researchers to target multiple genes simultaneously (Kabadi, Ousterout, Hilton & Gersbach, 2014; Sakuma, Nishikawa, Kume, Chayama & Yamamoto, 2014). CRISPR has emerged as powerful approach to precisely alter genome in potentially any living organism, including humans (Ceasar, Rajan, Prykhozhij, Berman & Ignacimuthu, 2016).**.** CRISPR locus is an array of acquired spacer interrupted by conserved repeated sequences. Following uptake, Invader foreign DNA is degraded by Cas9 nuclease and short nucleic acid foreign fragments (protospacers) are integrated in bacterial genome in the form of spacer sequences. Spacer motifs are variable depending on the length of

invading DNA. Next, spacer-repeat motifs are transcribed into CRISPR RNA (crRNA). CRISPR RNA (crRNA) associates with trans-activating CRISPR RNA (tracrRNA) to yield effector RNA complexes that recruit Cas9 endonucleases to the target sequences. Cas9 sgRNA ribonucleoprotein complex recognizes target sequence based on complementarity between crRNA and antisense strand of foreign DNA. Once Cas9 protein is recruited, it initiates interrogation process of target DNA (Sternberg, Redding, Jinek, Greene & Doudna, 2014) to search for Protospacer adjacent motif (PAM) sequence. PAM (Mojica, Diez-Villasenor, Garcia-Martinez & Almendros, 2009) is conserved ultra short sequence (2 to 3 nucleotides in length) positioned immediately adjacent or just one nucleotide apart from target sequence. After interaction with PAM sequence (Sternberg, Redding, Jinek, Greene & Doudna, 2014), Cas9 endonuclease becomes catalytically active and creates double strand breaks (DSB) resulting in degradation of foreign DNA. Cas9 nuclease possess two catalytic domains (Zhang, Wen & Guo, 2014), HNH nuclease and RuvC-like nuclease domains which cuts both crRNA complementary antisense strand and opposite sense strands, respectively, therefore creating Double stranded breaks in target DNA. Double stranded breaks (Figure 1.3) will elicit cellular DNA repair mechanisms, most commonly error prone- NHEJ, repair pathway that will introduce desired insertions, deletions or combination of insertions and deletions (indels). Insertions and deletions (indels) will create frameshift mutation that will disrupt the protein coding region of gene leading to specific gene knockout (Hsu, Lander & Zhang, 2014). The sequence specificity of CRISPR RNA (crRNA), existence of protospacer adjacent motif (PAM), intrinsic activity of Cas9 protein, and the relative abundance of sgRNA/Cas9 complexes relative to the target are among some factors that determine specificity and precision of CRISPR/Cas9 genome editing (Wu, Kriz & Sharp, 2014). CRISPR/Cas 9 can provide novel insight into the function of uncharacterized genes in mammalian cells via perturbation of gene screening. CRISPR/Cas9 technology offers promising potential to treatment of patients with cancers and inherited genetic diseases (Khan et al., 2016). One concern with CRISPR/Cas9 technique as with any loss of function gene screening studies is the occurrence of off-target effects. However, it has been demonstrated that the incidence of off-target effects is minimal with CRISPR/Cas9 platform compared to RNA interference (RNAi) technology (Smith et al., 2017).
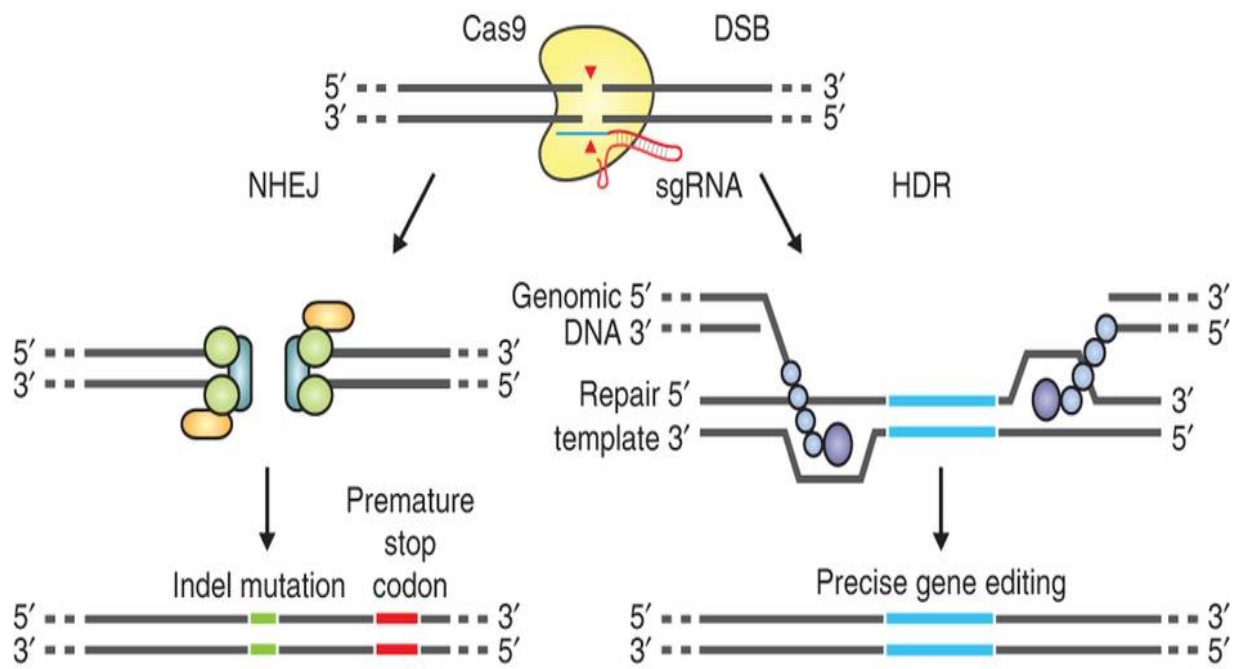
Figure 1.3: Proposed mechanisms of genomic editing of CRISPR/Cas9 platform.

Adopted from Sigma-Aldrich

1.8. Rationale for research work

Recent genome wide association studies have identified novel genetic susceptibility loci that are associated with essential hypertension in humans (Azam & Azizan, 2018; Johnson et al., 2011). For instance, LSP1/TNNT3 locus located on chromosome 11, containing genes encoding for human LSP1 and troponin T type 3. This finding was further supported by observation in our lab that adult LSP1 knockout mice have higher mean arterial blood pressure readings compared to their corresponding age matched wild type mice, and that endothelium nitric oxide-dependent vasodilatation in impaired in small resistance mesenteric arteries of LSP1 KO mice. Mechanism by which disruption of LSP1 gene contributes to hypertension and its associated vascular dysfunction is currently unknown. We believe that deficiency of endothelial LSP1 causes hypertension via alteration in the expression and activity of eNOS. To investigate our hypothesis, we have been utilizing LSP1 knockout mice as an in-vivo model and human endothelial cells lines (primary coronary artery endothelial cells and EA.hyb 926 cells) as an in-vitro model in order to fully understand the role of LSP1 in endothelial dysfunction.

1.9. Research Hypothesis

Our hypothesis is that LSP1 dictates endothelial function through modulation of expression and function of endothelial nitric oxide synthase.

1.10. Objectives of the study

1- Our main objective is to determine whether the knockout of LSP1 gene promotes endothelial dysfunction in animal model and human macrovascular endothelial cells. Furthermore, our goal is to elucidate the impact of LSP1 loss and gain of function on eNOS expression, eNOS coupling status, and nitric oxide production.

2- To reveal novel biochemical protein interaction of LSP1 and eNOS in mouse cardiovascular tissues and human endothelial cells.

1.11. Significance of the study

Hypertension is highly prevalent cardiovascular health condition that is estimated to affect 1.56 billion people by year 2025 (Kearney, Whelton, Reynolds, Muntner, Whelton & He, 2005). Hypertension is the strongest risk factor for ischemic heart disease, cerebrovascular accidents, renal failure, cardiac arrhythmias, valvular heart disease, and left ventricular hypertrophy, which are the leading sources of cardiovascular morbidity and mortality (Kjeldsen, 2018; Vasan et al., 2002). Nitric oxide (NO) synthesized by eNOS has central role in regulation of arterial blood pressure and NO deficiency in considered as prominent feature of endothelial dysfunction in hypertension (Li, Youn & Cai, 2015). Deep comprehension of molecular signalling pathways of hypertension is crucial for designing novel treatment strategies. With the exception of statins, no current drug therapies can directly increase the expression of eNOS. Our study has unveiled the new role of LSP1 in regulation of eNOS expression and may facilitate identification of novel drug targets.

2. MATERIALS AND METHODS

2.1. Cell culture

 EA. hy926 cells (immortalized human umbilical vein endothelial cells) were purchased from American Tissue Culture Collection (Rockville, MD, USA) and were grown in Dulbecco's modified Eagle medium (Cellgro, VA, USA) supplemented with 10% Fetal bovine serum (Hyclone, UT, USA), 100 U/mL penicillin and 100 µg /mL streptomycin (Hyclone), and HAT supplement (Invitrogen) and were maintained in 5% CO_2 incubator at 37 °C. Cells between passages 2 and 6 were used for experiments. Primary human coronary artery endothelial cells (HCAEC) were obtained from Cell Applications (San Diego, CA, USA), and were cultured in MesoEndo growth medium (Cell Applications) in 5% CO_2 incubator at 37 °C. For subculturing of primary cells, cells were detached by Trypsin-EDTA solution (Cell Applications) at room temperature, and the activity of trypsin was halted when cells become rounded by the addition of Trypsin-neutralizing solution (Cell Applications). Cells were then centrifuged at $200 \times g$ for 5 minutes followed by resuspension in MesoEndo growth medium. Cells in passages 2 to 4 were utilized for experiments.

2.2. Plasmid transfection

EA. hy926 cells cultured in 12 well plate were transfected with 1µg of either CRISPR sgRNA Cas9 lentivector targeting specific regions of human LSP1 or scramble control sgRNA Cas9 vector using Cytofect Endothelial Cell transfection kit (Cell Applications). Transfection process was performed when endothelial cells reached 80% confluency in presence of serum-containing DMEM medium. Transfection complex was prepared by mixing 1 µg lentivector, 5µl Cytofect II, 3µl peptide enhancer in 250µl of transfection medium (serum-free antibiotic-free growth medium) followed by incubation at 37 °C for 25 minutes. Cytofect II is non-lipid-based cationic transfection reagent. Viral enhancer improves intracellular plasmid delivery by utilizing adenoviral receptors on the cell surface. Peptide enhancer increase transfection efficiency by escorting CF II-DNA complex to the nucleus. Transfection cocktail was added to 1mL of serum-containing DMEM in each well of 12 well plate. Transfection medium was aspirated next morning and replaced with antibiotic-free

growth medium. Transfected endothelial cells were harvested after 120 hours for analysis of gene expression of human LSP1 and eNOS.

2.3. Lentiviral packaging

Three LSP1 sgRNA CRISPR/Cas9 All-in-One Lentivectors (pLenti-U6-sgRNA-SFFV-Cas9-2A-Puro, Catalog Number: K1242305), and one scramble sgRNA CRISPR/Cas9 All-in-One Lentivector (pLenti-U6-sgRNA-SFFV-Cas9-2A-Puro, Catalog Number: K010), were purchased from Applied Biological Materials (Abmgood, Richmond, BC, Canada). Two LSP1 sgRNA CRISPR/Cas9 lentivectors targeting 20 base sequences beginning at **12** (**5'** TTCGAGTGACCCGGGTGCCG **3'**), and at **208** (**5'TCCAGTTCAGGGGCCTCCGA 3'**) of human LSP1 gene were used in this study. Replication incompetent lentiviruses were generated in HEK293T cells (at 90% confluency in 100mm dish) by transient co-transfection of third generation lentiviral packaging plasmids (GAG-POL, REV, and VSV-G plasmids) and CRISPR sgRNA Cas9 encoding lentivectors using lentifectin transfection reagent (all purchased from Applied biological materials) as per manufacturer protocol. Lentiviral supernatants were harvested twice every 48 hours thereafter, concentrated by Lenti-X Concentrator (Clonetech, Heidelberg, Germany) and the appropriate lentiviral titer was determined by Lenti-Gostix kit from (Clontech).

2.4. Lentiviral transduction and Western Blotting

EA. hy926 cells were transduced with lentiviruses encoding for CRISPR sgRNA/Cas9 inserts targeting human LSP1 and scramble control lentivirus at multiplicity of infection (MOI) of 2 in the presence of 12 µg/mL polybrene infection reagent (EMD Millipore) and 10mM HEPES (Hyclone). Viral supernatant was left on the cells until they ready for subculture. Forty-eight hours post transduction, the cells were split at 1:3 ratio and were subjected to 2nd round of lentiviral infection. Eight to twelve days post-lentivirus transduction, the cells were harvested by cell scraper and lysed in RIPA buffer supplemented with protease and phosphatase inhibitors. Equal amounts of protein (30 µg) were loaded and resolved on 10% SDS PAGE gels followed by semi-dry transfer to nitrocellulose membrane. The membranes were then blocked with 5% skimmed milk in TBST and probed with rabbit polyclonal anti-human LSP1 antibody (Santa-

Cruz; catalog number. SC-33160), mouse monoclonal anti-eNOS antibody (BD Biosciences; catalog number. 610297), mouse monoclonal anti-GAPDH (Origene; catalog number. TA802519), and mouse monoclonal anti- βeta actin (Santa-Cruz; catalog number; catalog number. SC-47778.).

2.5. Real time quantitative PCR

One-week post-lentivirus transduction, total cellular RNA was extracted from control and LSP1 KO human endothelial cells by Nucleozol extraction reagent (Clontech). Appropriate RNA concentration and purity was determined by Nanodrop spectrophotometer. A total of 1μg cellular RNA was used for each Reverse transcription reaction, and first strand cDNA synthesis was performed via Oligo-deoxythymine (dT) priming using Quantibio Flex cDNA synthesis kit (Quantibio) as per manufacturer instructions. Quantitative real-time amplification cycles were performed in Bio-Rad CFX 96 Thermocycler utilizing PerfeCTa SYBR Green SuperMix (Quantabio). The reactions were run in a total volume of 50 μl at the following temperature settings: Denaturation of cDNA template: 95°C for 3 min, PCR cycling (40 cycles:) 95°C for 15 s and 52°C (primer annealing) for 45 s, followed by melting curve dissociation phase. The primers set for housekeeping gene (human HPRT1) and target gene (eNOS human) were used for each amplification reaction and were purchased from Origene. The melting curve was illustrated as the peak of the negative derivative of fluorescence temperature graph. Data from real-time PCR reactions were analyzed by Delta-Delta CT (Livak method). The relative changes in eNOS mRNA abundance were assessed by the following equations.

ΔCT (control) = CT (eNOS, control) – CT (HPRT, control)

ΔCT (experimental, LSP1 KO) = CT (eNOS, experimental) – CT (HPRT, experimental)

Then, the ΔCT of the experimental (LSP1 KO) samples were normalized to the ΔCT of the calibrator (control sample): $\Delta\Delta$CT = ΔCT (test) – ΔCT (calibrator).

Finally, Relative quantification = $2^{-\Delta\Delta CT}$.

2.6. Measurement of intracellular nitric oxide levels

Nitric oxide synthesis was measured by fluorescent staining with DAF-FM diacetate (cell-permeable nitric oxide sensitive indicator, ThermoFisher Scientific) at working concentration of 5μM in EA.hy926 cells. The Cells were incubated with fluorescent probe and the stimulus (Thapsigargin at final concentration of 100 nM) in Krebs buffer for 1 hour in 5% CO_2 incubator at 37 °C followed by wash twice with PBS to remove excess fluorescent probe. The fluorescent microscopic images were acquired under excitation/emission wavelength (495/515) by Bio-Rad Zoe fluorescent inverted cell imager. DAF-FM mean fluorescence intensities were analyzed by ImageJ software, and statistical analysis was performed by GraphPad Prism Software.

2.7. Coimmunoprecipitation

For coimmunoprecipitation of native proteins, EA. hy926 cells were grown in 100mm dishes until they reach confluence, washed twice with ice-cold PBS and were scraped and lysed in CHAPS immunoprecipitation buffer supplemented with protease and phosphatase inhibitors. Protein lysates were transferred to 1.5 ml Eppendorf tubes, were hold for 30 minutes on ice, and were mixed by gentle repeated inverting and pipetting until samples were homogenous. Insoluble material was cleared by centrifugation at 12000 × g for 10 minutes at 4°C. Fraction of protein supernatant was utilized as input, while remaining cellular lysate was used for coimmunoprecipitation. Equal amounts of cleared protein extracts were incubated with 8 μg of either anti-eNOS rabbit polyclonal antibody (Abnova Corporation, Taipei, Taiwan.; catalog number. PAB12680) or non-immune rabbit IgG (Cell signalling; catalog number. 2729S) overnight at 4°C with constant rotation. Then 50μl of PureProteome protein A/G (EMD Millipore) were added to immobilize antigen-antibody complexes and were incubated for 1 hour at room temperature with end-to-end rotation. The beads were collected with magnetic stand, were washed three times with PBST, and protein complexes were eluted off beads by heating in hot SDS sample buffer without reducing agent at 95°C for 10 minutes. After that, DTT reducing agent (50 mM) was added to the elution fractions, and immunoprecipitated endogenous LSP1-eNOS protein complexes were resolved by SDS-PAGE gel, transferred to immobilon-E PVDF membranes (EMD Millipore), and probed with mouse monoclonal anti-eNOS antibody and rabbit polyclonal anti-LSP1 antibody. To minimize the detection of denatured immunoglobulin heavy chain which has

approximately molecular weight of 55 kD, positioned just below human LSP1 in SDS-PAGE gel, and which can interfere with detection of endogenous human LSP1 signal, Tidy blot specific horseradish peroxidase (HRP) conjugated secondary antibody (Bio-Rad) at 1:200 dilution was utilized instead of conventional secondary HRP conjugated anti-rabbit antibodies.

For coimmunoprecipitation of exogenous LSP1, mammalian expression construct coding for human LSP1 tagged with turbo-GFP at the C-terminus was transiently transfected into EA. hy926 cells seeded in 100mm cell culture dish using Xfect transfection reagent (Clonetech). Seventy-two hours post-transfection, stably transfected cells were selected in the presence of G418 at concentration of 500 µg/mL (Invivogen). G418 resistance was conferred by the presence of neomycin resistance gene within the vector. After three weeks, neomycin resistant colonies were expanded and maintained in DMEM and characterized for expression of tGFP-tagged LSP1. For coimmunoprecipitation, stably transfected cells were grown in 100mm cell culture dishes until confluence, washed twice with ice-cold PBS, scraped, and lysed in NP40-based non-denaturing lysis buffer (Pierce IP buffer, Thermofisher Scientific) supplemented with protease and phosphatase inhibitors. After 30 minutes incubation on ice with intermittent pipetting, crude cell lysates were centrifuged at $12,000 \times g$ for 10 minutes at 4°C. Protein supernatant was collected and incubated overnight with 10µg anti-turbo GFP antibody (Origene; catalog number. TA150041) at 4°C. On the following day, 50µl of Dynabeads Protein G (ThermoFisher Scientific) was added to antibody protein mixture and incubated for 30 minutes with constant rotation at room temperature to capture immune-complexes. Then, Dynabeads were washed three times with PBST to eliminate non-specific binding, and protein complexes were eluted by incubation in hot non-reducing SDS lammeli buffers at 95 °C for 10 minutes. The protein elutes were reduced by 50 mM DTT prior to loading on SDS-PAGE gels.

2.8. Adenovirus packaging and transduction

Human LSP1 HA adenovirus under the control of cytomegalovirus (CMV) promoter and control CMV null adenoviruses were purchased from Applied Biological Materials. HEK293 cells were cultured in minimal essential medium (ATCC) supplemented with 10% FBS (Hyclone) and were utilized for adenovirus amplification. Adenoviral particles were harvested when 95% of cells were

detached from dishes and were subjected to three freezing/thawing cycles followed by centrifugation at $1500 \times g$ for 10 minutes at room temperature to pellet cell debris.

For adenovirus transduction, EA.hy926 cells were seeded at 60% confluency one day prior to transduction in 100 mm cell culture dishes and were infected with 5mL of LSP1 HA and CMV null adenovirus supernatants for 24 hours. The protein expression of human LSP1 and eNOS in endothelial cell lysates was determined at 48 to 72 hours following time of initial adenovirus transduction by immunoblotting.

2.9. Confocal immunofluorescence microscopy

EA. hy926 cells were plated in eight chamber poly-lysine D tissue culture treated glass slides (BD Falcon). The media was rinsed with ice-cold PBS and the cells were fixed in 4% paraformaldehyde in PBS. Fixed cells were permeabilized in 0.3% Triton X-100 for 5 minutes, washed twice for 5 minutes each with PBS, and then blocked with 8% bovine serum albumin (BSA) for 30 minutes at room temperature. The cells were incubated with rabbit polyclonal LSP1 and mouse monoclonal eNOS primary antibodies in 1% BSA overnight at 4°C, followed by incubation for 2 hours at room temperature with Alexa Fluor 647-labelled donkey anti-rabbit and Alex Fluor 488-labelled goat anti-mouse secondary fluorescent probes (Molecular Probes. Invitrogen), respectively. For colocalization of exogenous LSP1 with eNOS, immunofluorescence staining was performed in stably transfected EA. hy926 endothelial cells expressing human LSP turbo-GFP fusion protein by incubation of cells with mouse monoclonal anti-eNOS antibody only and followed by detection with Alexa Fluor 568-labelled donkey anti-mouse secondary antibody. The glass slides were mounted with prolong Gold antifade reagent with DAPI (Invitrogen), and cells were visualized with Zeiss LSM700 inverted confocal microscopy system.

2.10. Determination of eNOS dimerization status

Aorta tissues from both WT and LSP1 KO mice (14–16 weeks old) were homogenized by cryogrinding in Pierce IP lysis buffer (mild non-denaturing NP40-based lysis solution). Protein extracts were not subjected to boiling step and were mixed with 4X Laemmli sample buffer without reducing agent on ice. The protein samples were loaded on 7% SDS gel and separated using low-temperature SDS-PAGE as previously described (Benson et al., 2013). Gels and running buffers were equilibrated to 4 degrees Celsius before electrophoresis and the temperature was maintained at 4°C by placing Gel tank in ice during electrophoresis. eNOS dimers and monomers were detection by incubating blots with mouse monoclonal anti-eNOS antibody (BD-Bioscience).

2.11. Experimental Animals studies

Male WT SVJ129 (control) and LSP1 (12 to 16 weeks) mice were used in our studies. The mice were anesthetized (60 mg/kg per body weight intraperitoneal pentobarbital) and euthanized. The whole aorta and mesenteric arteries were carefully removed and dissected free from perivascular fat connective tissue in cold (4°C) DMEM under dissecting microscope and were immediately frozen in liquid nitrogen and stored in -80 °C freezer until testing. For study of eNOS expression levels, aortae and mesenteric arteries from both WT and LSP1 mice were homogenized by cryogrinding in ice-cold RIPA buffer supplemented with protease and phosphatase inhibitors. The tissue homogenates were cleared from insoluble material by centrifugation at 17000× g for 20 minutes at 4°C. The clarified protein extracts were analysed by immunoblotting for expression of eNOS and immunoblots were probed with mouse monoclonal anti-eNOS antibody raised against C-terminus of the enzyme (BD-Bioscience), mouse monoclonal anti-βeta actin antibody (Santa-Cruz), and rabbit polyclonal anti-eNOS antibody (Abnova). All animal procedure were approved by the University Committee on Animal Care and Supply (UCACS) of the University of Saskatchewan and met the standards of the Canadian Council on Animal Care.

2.12. Statistical analysis

All western blotting bands were quantified using Image Lab software (Bio-Rad). Analysis of statistical significance was performed in GraphPad Prism version 5 software utilizing One-way ANOVA and student t-test. All data sets were expressed as mean $\pm$ SEM. P value level < 0.05 was considered statistically significant.

3. RESULTS

3.1. CRISPR/Cas9 mediated genomic editing of human LSP1 gene in EA.hy926 cells and primary HCAECs

In order to disrupt the protein expression of human LSP1 gene in EA.hy926 cells and primary HCAECS, specific guide RNAs Target 1 and 3 were engineered to target exon 1 of human LSP1 gene at the following sequences, starting at **12** (5' TTCGAGTGACCCGGGTGCCG 3'), and at **208** (5'TCCAGTTCAGGGGCCTCCGA 3'), respectively. CRISPR/Cas9 lentivectors encoding exon 1-specific guide RNAs were transfected into EA.hy926 cells via cytofect endothelial cell transfection (Cell Applications). Although EA.hy926 cells are difficult to transfect cell line, we have utilized novel non-lipid cationic transfection reagent (Cytofect II) which has been previously reported to achieve 70 to 80% transfection efficiency in primary HUVEC (Walia, 2011). Because CRISPR/Cas9-mediated knockdown is initiated at the level of DNA, unlike RNAi knockdown tools (siRNA and shRNA) which work at the level of messenger RNA, and since most CRISPR/Cas9-induced Indels mutations occur 5 to 10 days post lentiviral transduction (Yuen et al., 2017), we opted to harvest endothelial cells and screen for LSP1 knockout by immunoblotting at least 7 to 12 days post lentiviral transduction to allow sufficient time for Indel mutations to occur in human LSP1 gene, and for human LSP1 protein abundance to go down by significant degree. For plasmid delivery of sgRNA/Cas9 lentivectors, because sgRNA/Cas9 expression is driven by strong RNA polymerase III-dependent U6 promoter, we have harvested endothelial cells at earlier time points compared to viral delivery of CRISPR/Cas9, between 72 to 120 hours after transfection, and we have achieved the most significant knockdown at 120 hours post transfection. To markedly enhance efficiency of lentiviral transduction, we have subjected endothelial cells to two rounds of infection with sgRNA/Cas9 lentiviruses as previously reported (Gong, Liu, Klomp, Merrill, Rehman & Malik, 2017) .In our results, we have confirmed successful CRISPR/Cas9 genomic editing by immunoblotting, and we have achieved very significant knockdown of human LSP1 both in EA.hy926 cells and primary HCAECs (Figures. 3.1A, 3.2A *middle panel*, and Fig. 3.3 *lower panel*). Our data indicate that knockdown is mostly apparent at the expected molecular weight of human LSP1 (60kD) from previous reports (Huang, Zhan, Ai & Jongstra, 1997; Pulford,

Jones, Banham, Haralambieva & Mason, 1999). However, we have also observed significant knockout at molecular weight of 55Kd (Figures 3.1A, 3.2A, *middle panel*). Furthermore, this 55 kD protein most likely represent another isoform of human LSP1 detected by rabbit polyclonal anti-human LSP1 antibody. Remarkably, earlier study (Carballo, Colomer, Vives-Corrons, Blackshear & Gil, 1996) has confirmed that human LSP1 is indeed two proteins with apparent molecular weights of 60 and 55 kD. Furthermore, 55 and 60 kD proteins are both LSP1 and difference in molecular weight in due to posttranslational modification (phosphorylation).
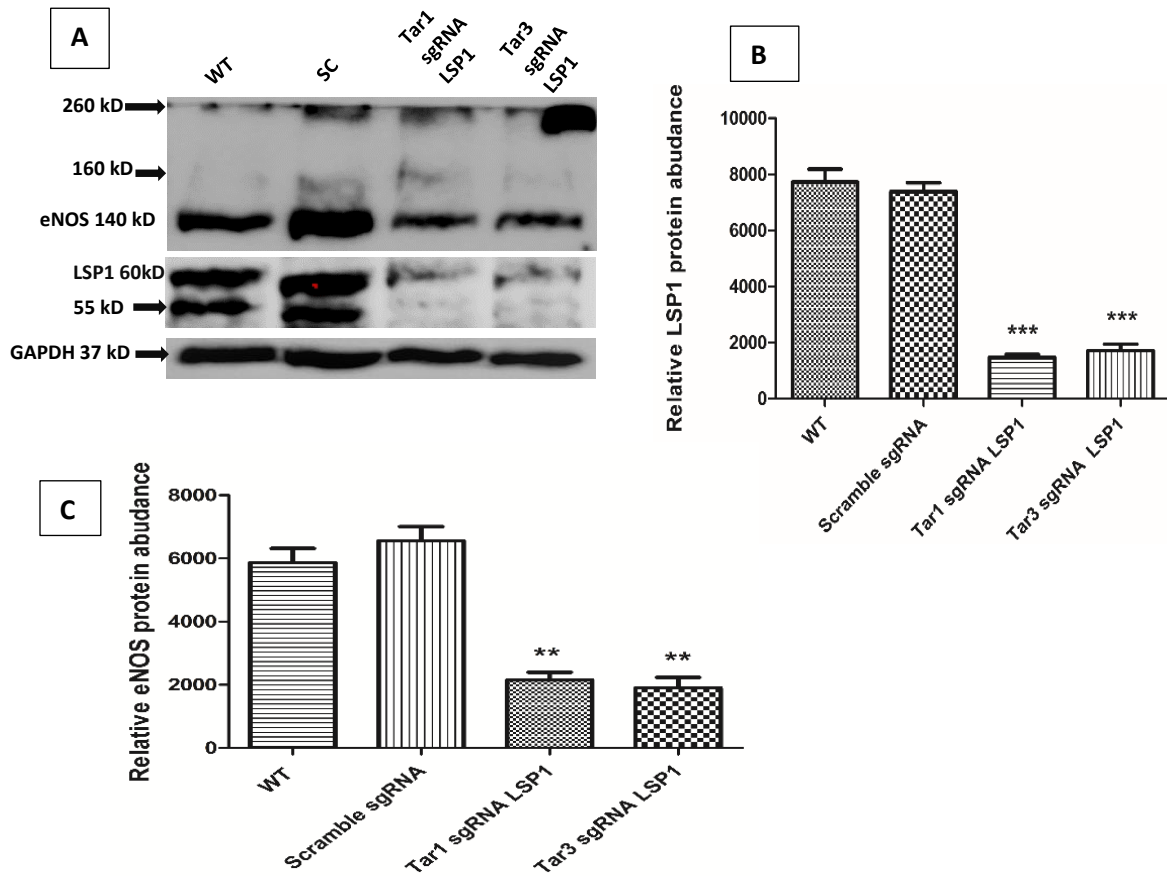
Figure 3.1: Knockdown of human LSP1 gene in EA. hy926 cells by plasmid-mediated delivery of CRISPR sgRNA/Cas9 lentivectors. Cultured EA. hy926 cells were transiently transfected with sgRNA Cas9 all-in-one lentivectors against two distinct domains of human LSP1 gene (sgRNA targets 1 and 3) or SC sgRNA Cas9 all-in-one lentivector via cytofect II transfection reagent. (**A**) Total cellular lysate (30 μg /lane) of wild type (WT), scrambled control (SC), Tar1 and Tar3 sgRNA KO groups were resolved by SDS-PAGE and protein expression of eNOS, human LSP1 and GAPDH loading control was revealed by immunoblotting. Tar1 and Tar3 sgRNA LSP1 groups are two specific KO groups created by CRISPR/Cas9-mediated genomic editing of human LSP1 gene at two peculiar sequences (two different sgRNAs). (**B**) and (**C**) Quantification analysis of LSP1 60kD and eNOS protein levels in EA. hy926 endothelial cells 4 days post-transfection, respectively. Data are presented as mean ± SEM from 3-independent experiments, n=3. P value was measured by student t test by comparing each knockdown sgRNA group to scramble control sgRNA. ** denotes p value < 0.01, *** p value < 0.001.
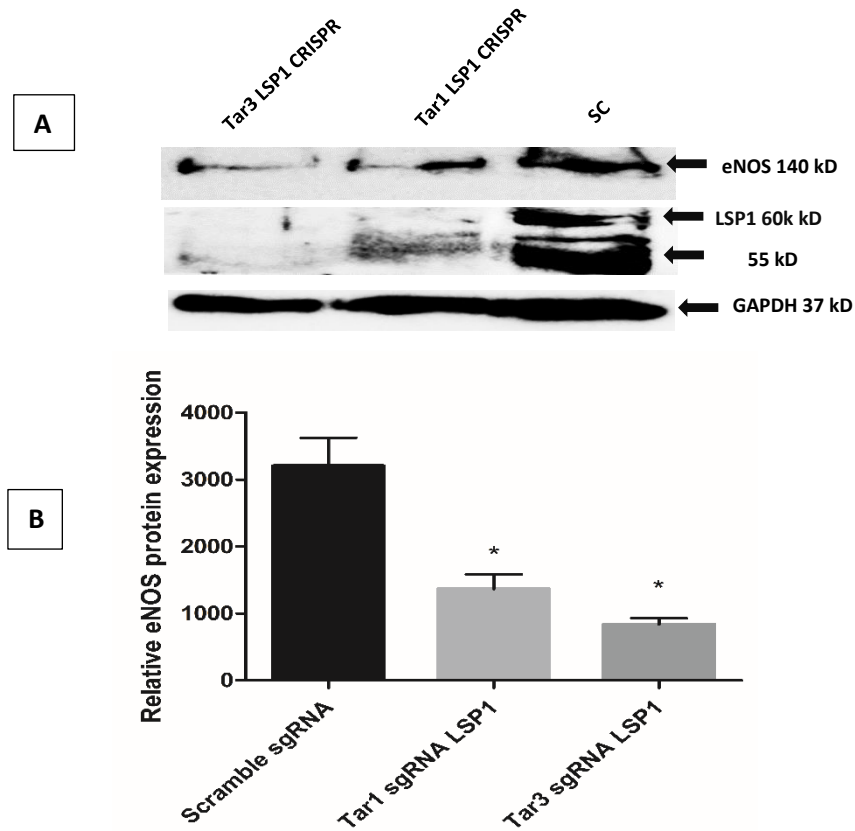
Figure 3.2: Knockdown of human LSP1 in primary HCAECs utilizing CRISPR-Cas9 technology. Cultured primary HCAECs were transduced with sgRNA Cas9 all in one lentiviruses against two specific sequences of human LSP1 gene (sgRNA targets 1&3) or SC sgRNA Cas9 all-in-one lentivirus at multiplicity of infection of 2. (**A**) Protein expression of human LSP1 and eNOS was determined by immunoblotting eight days post-lentivirus transduction. (**B**) Densitometry analysis of eNOS protein abundance from two independent experiments is shown, $n=2$. * denotes $p < 0.05$ comparing each knockout group to scramble control.
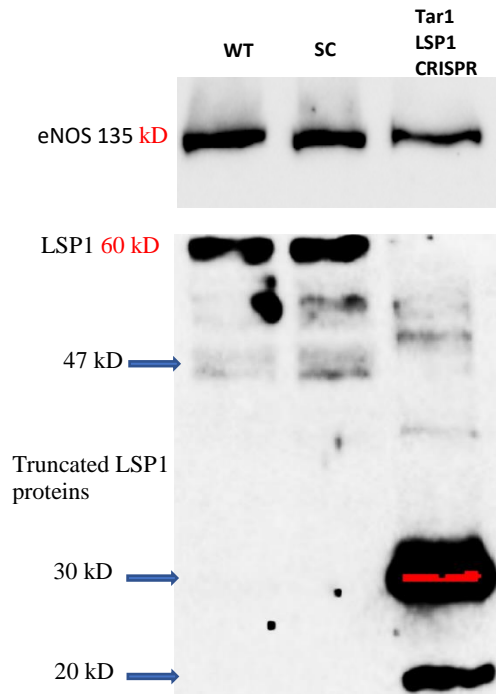
Figure 3.3: Exon skipping and formation of truncated LSP1 proteins in one CRISPR mutated polyclonal cells sample. Original uncropped immunoblot from CRISPR edited EA.hy926 cells revealed a unique phenomenon, which is the formation of aberrant truncated human LSP1 proteins. This unexpected consequence has been recently reported in the literature as result of exon skipping. Furthermore, this aberrant truncated LSP1 proteins do not rescue the phenotype of endothelial dysfunction (manifested by downregulation of eNOS expression) that result from loss-of-function of full length functional LSP1. The immunoblot has been probed with rabbit polyclonal LSP1 antibody raised against almost full-length human protein. The antibody has detected truncated LSP1 proteins in 2 independent experiments, *n*=2.

3.2. Knockdown of human LSP1 reduces eNOS gene expression in EA.hy926 cells and primary HCAECs

To address our hypothesis that LSP1 is crucial in regulation of eNOS gene expression, we have measured gene expression of eNOS at transcriptional and protein levels in endothelial cells in which LSP1 was depleted by CRISPR/Cas9 lentivectors. As shown in Figure 3.4A, knockdown of human LSP1 induced significant reduction in the abundance of eNOS mRNA in EA.hy926 cells by more than 80% downregulation for Tar1 (p value < 0.0001, n=4) and more than 70% for the Target 3 (p value < 0.0001, n=4) sgRNA LSP1 KO groups, respectively. We have chosen HPRT (hypoxanthine guanine phosphoribosyl transferase) gene instead of GAPDH as reference control in our RT-PCR analysis based on recommendation from recent studies which establish a set of stably expressed genes for accurate normalization of RT-PCR data in human endothelial cells (Chen et al., 2013; Zyzynska-Granica & Koziak, 2012). Furthermore, HPRT was highly stable in our RT-PCR data and its expression was not influenced by knockout of human LSP1. We further demonstrated that depletion of human LSP1 via CRISPR/Cas9 technology caused considerable decrease in eNOS expression at the protein level in EA.hy926 cells (more than three-folds downregulation for both Tar1&3 sgRNA LSP1 KO groups (Fig 3.1A, *upper panel*, p value < 0.01, n=3). We have also confirmed the downregulation of eNOS at protein level following knockdown of human LSP1 in endothelial cells of different origin (Primary HCAECs, Figure 3.2A, *upper panel*). The changes in eNOS protein expression seems to reflect changes in eNOS mRNA levels (Figure 3.1A and 3.4A). To exclude the possibility of off-target effects on human eNOS gene by LSP1 CRISPR/Cas9 lentivectors, we have verified the reduction in eNOS gene expression with two different CRISP/Cas9 lentivectors targeting two specific sequences of human LSP1 gene (sgRNA Cas9 targets 1 and 3), and we have observed comparable changes in eNOS mRNA and protein abundance. The trend of reduction of eNOS gene expression seems to reflect changes in LSP1 protein levels (Figure 3.2A). To our surprise, some CRISPR mutated clones have escaped the mutation and synthesize truncated LSP1 protein fragments at very high expression levels (Figure 3.3, *lower panel*). Although this was unexpected phenomenon, there have been several reports in literature suggesting that exon skipping could indeed occur following CRISPR/Cas9 mediated genome editing (Chen, Tang, Li, Hou, Wang & Kang, 2018; Mou et al., 2017; Sharpe & Cooper, 2017). Intriguingly, mutations following CRISPR/Cas 9 genome editing have been demonstrated to result in alternative splicing of pre-mRNA and formation of truncated proteins

revealed by immunoblotting (Kapahnke, Banning & Tikkanen, 2016). However, to our knowledge, such strong expression of truncated proteins following CRISPR genome editing has not been previously reported in literature and could suggest that LSP1 has very significant role in signal transduction. While formation of truncated LSP1 protein fragments might slightly rescued eNOS expression (Figure 3.3, *upper panel*), suggesting that truncated LSP1 proteins might act in dominant positive manner. However, truncated LSP1 proteins did not restore expression of eNOS back to normal levels, implying that the presence of full-length LSP1 protein is essential for expression of normal levels of eNOS in human endothelial cells.
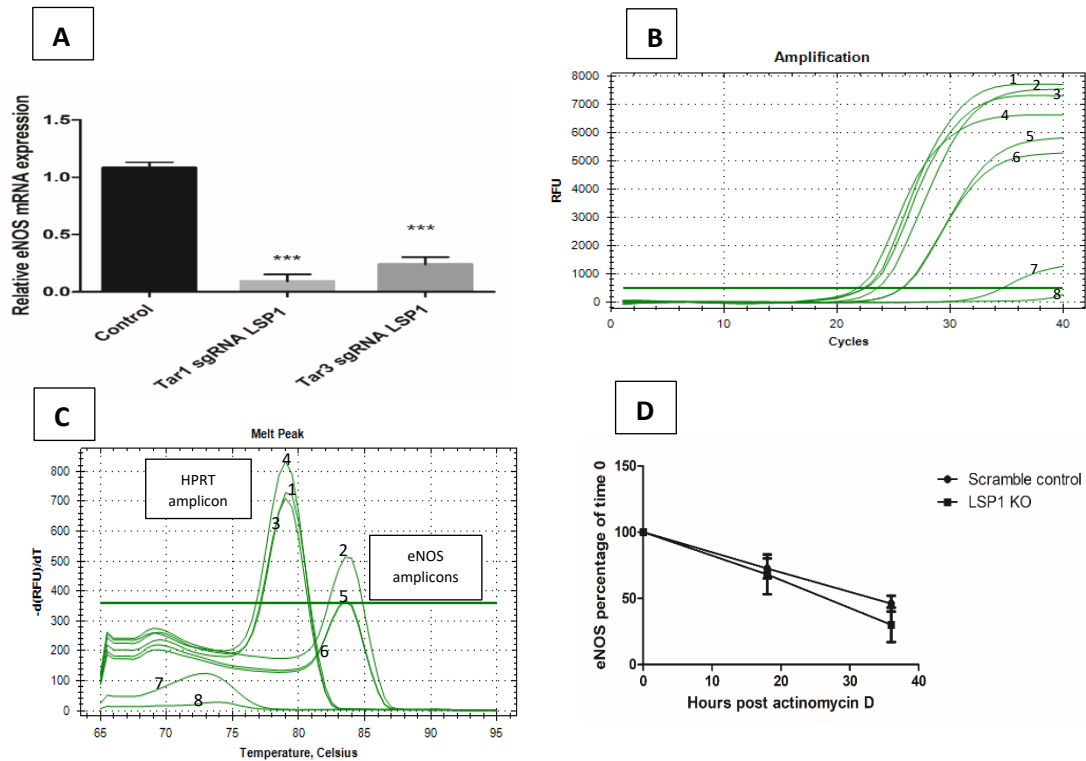
Figure 3.4: The relative expression of eNOS mRNA in EA.hy926 cells following knockdown of human LSP1. EA.hy926 cells were transduced with sgRNA/Cas9 lentiviruses Targets 1&3, and SC sgRNA/Cas9 lentivirus. Total cellular RNA was extracted, reverse transcribed, and 1/10th of the resulting complementary DNA was used as template for each quantitative real time PCR reaction. (**A**) The relative expression of eNOS mRNA was determined by delta-delta CT (Livak method) as described in *Materials and Methods*. Data represent mean ± SEM of four biological replicates, *n*=4. *p* value < 0.0001 (one-way ANOVA, Tar1&3 vs control). (**B**) Amplification curves corresponding to eNOS and HPRT amplicons obtained SC and LSP1 KO Tar1 and Tar3 CRISPR groups. (**C**) Melt curves of eNOS and HPRT amplicons demonstrating specificity of amplification. Numbers: 1,2,3,4,5, and 6 illustrate (HPRT control, eNOS control, HPRT Tar3 sgRNA, HPRT Tar1 sgRNA, eNOS Tar3 sgRNA, and eNOS Tar1 sgRNA) amplicons, respectively. Negative controls (non-template controls) for eNOS and HPRT1 are highlighted by numbers 7 and 8, respectively. *** denotes *p* value < 0.001 by student t test comparing each LSP1 KO group to scramble control group. (**D**) Stability of eNOS mRNA following knockdown of human LSP1. Control and LSP1 depleted EA.hy926 cells were treated with transcriptional inhibitor (actinomycin D) at concentration of (2.5 μg /mL) for 0, 18, and 36 hours. Endothelial cells were harvested and eNOS mRNA abundance was determined at each time point by RT-PCR analysis. Data are mean ± SEM of two biological replicates, n=2. p value > 0.05.

3.3. Human LSP1 overexpression enhances the expression of eNOS

To further examine whether endothelial LSP1 has a positive role in regulation of eNOS transcription, we tested whether overexpression of human LSP1 would lead to increased eNOS expression. For this purpose, we have utilized adenoviruses to obtain robust transient expression of human LSP1. We have employed adenoviral vector in which expression of human LSP1 is driven by strong CMV promoter, and we have transduced endothelial cells with CMV-LSP1 HA adenovirus or CMV-null adenovirus (adenovirus with CMV promoter only without coding sequence) as the control. As shown in Figure 3.5 (*middle panel*), we have obtained the strongest expression of human LSP1 at 48 hours after time of initial adenoviral transduction. Overexpression of human LSP1 via adenoviruses has markedly increased expression of eNOS (more than 4 folds upregulation at 48 hours post LSP1-HA adenovirus transduction) compared to CMV-null adenovirus control at 48 hours post CMV-null control adenovirus transduction (Figure 3.5A, *upper panel*). Remarkably, in similar trend to CRISPR/Cas9 mediated knockdown of human LSP1, upregulation changes in eNOS expression levels were correlated with increases in LSP1 protein abundance (Figure 3.5A, *middle and upper panels*) and was most statistically significant at 48 hours post adenovirus transduction (*p* value=0.006).
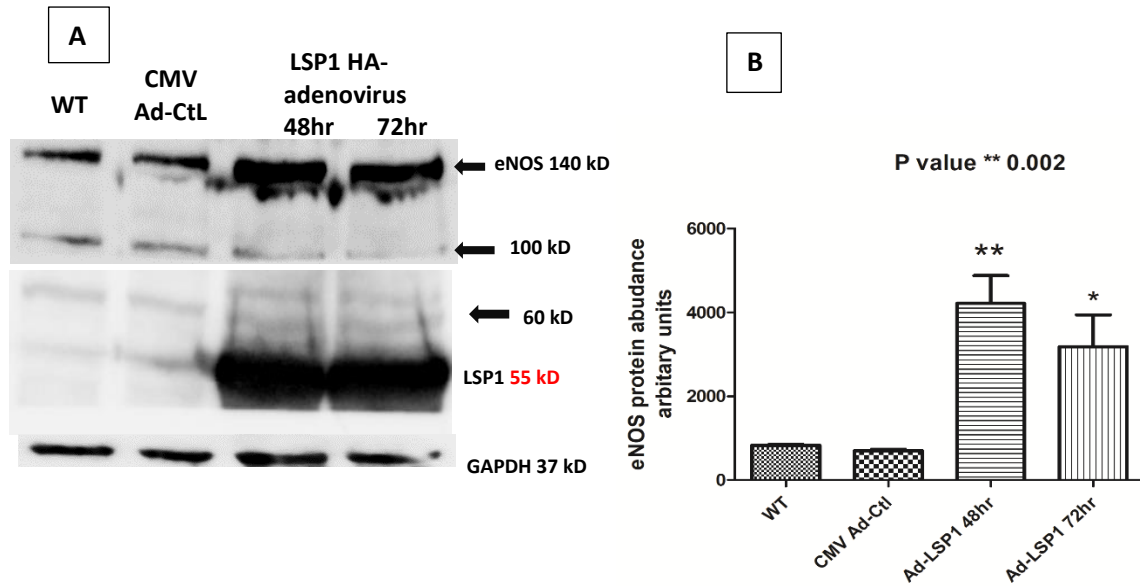
Figure 3.5: Overexpression of human LSP1 via adenoviral vector upregulates the expression of endothelial nitric oxide synthase. EA. hy926 cells seeded in 100 mm cell culture dishes were transduced with LSP1-HA adenovirus or control CMV null adenovirus for 24 hours. (**A**) The protein expression of human LSP1, eNOS, and GAPDH loading was determined at 48- and 72-hours following time of initial adenovirus transduction by immunoblotting. (**B**) Quantification analysis of eNOS protein expression in wild type (WT), CMV-null adenovirus control (CMV Ad-Ctl), and LSP1-HA adenovirus transduction groups at 48 and 72 hours (Ad-LSP1 48&72 hr). Statistical analysis was performed by one-way ANOVA followed by student t test. p value =0.002 between groups (LSP1 HA adenovirus groups vs CMV null adenovirus control) by one way-ANOVA. * denotes $p < 0.05$, ** $p < 0.01$ by t test comparing each LSP1-HA adenovirus overexpression group to CMV-null adenovirus group. Data are representative of three independent experiments, n=3.

3.4. Human LSP1 and eNOS interact physically in human macrovascular endothelial cells

To determine whether human LSP1 interact physically with eNOS, we first have performed coimmunoprecipitation in EA.hy926 cells under non-denaturing conditions. EA.hy926 cells were lysed in CHAPS IP lysis buffer under basal physiological conditions, and lysates were then subjected to immunoprecipitation with rabbit polyclonal anti-eNOS antibody. Subsequently, eNOS immunoprecipitates were analyzed by immunoblotting and were probes with anti-LSP1 rabbit polyclonal antibody and anti-eNOS mouse monoclonal antibody. As shown in Figure 3.6A (*lower panel*), LSP1 was successfully and specifically immunoprecipitated in anti-eNOS elutes fractions. We examined the specificity of the interaction of two proteins by utilization of non-immune rabbit IgG in place of anti-eNOS antibody and by probing for other protein (ERM protein) that is not anticipated to interact with eNOS (data not shown here). As seen in the lane of post-IP fraction (Figure 3.6A), eNOS was barely detectable, implying successful immunoprecipitation of majority of eNOS protein complexes, whereas significant portion of LSP1 was still unbound, suggesting that there was fraction of endothelial LSP1 not interacting with eNOS. Because coimmunoprecipitation technique involves the use of detergents and washing steps that may disrupt protein-protein interactions, it is difficult to ascertain how much of cellular pool of LSP1 associates with eNOS.

To provide additional evidence of LSP1 binding to eNOS, we have transfected human LSP1-tGFP cDNA construct into EA.hy926 cells and we have generated stable endothelial cell line permanently expressing tGFP-LSP1 fusion protein. Next, we immunoprecipitated tGFP-LSP1 protein complexes from endothelial cell lysates with anti-tGFP mouse monoclonal antibody and probed the immunoblots with anti-eNOS mouse monoclonal antibody. eNOS was successfully and specifically immunoprecipitated by anti-tGFP antibody (Figure 3.7, *upper panel*).
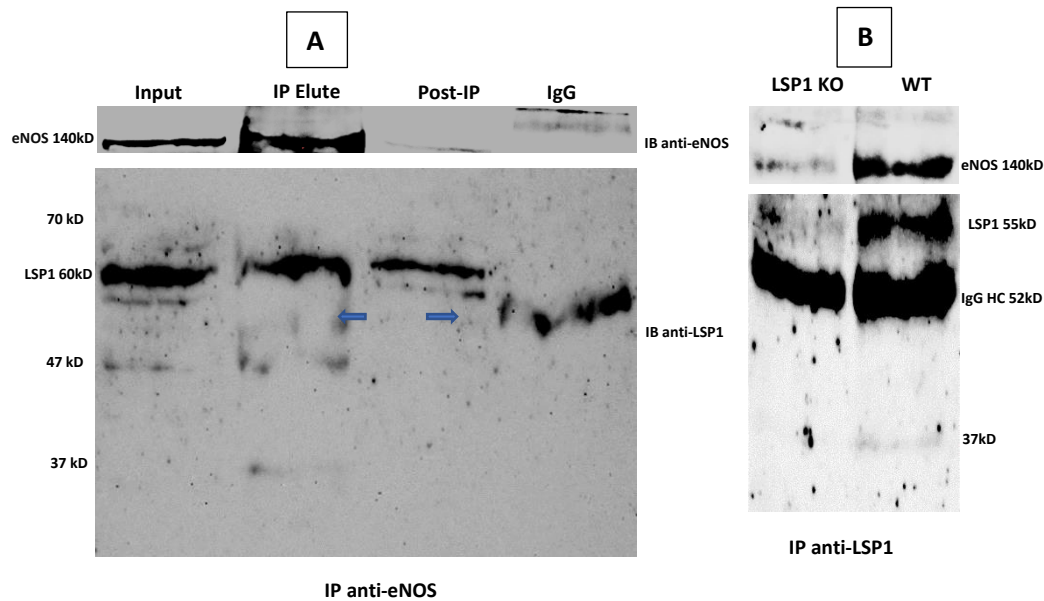
Figure 3.6: Physical interaction between eNOS and LSP1 in human macrovascular endothelial cells. (**A**) Representative immunoblotting analysis of eNOS immunoprecipitates. EA.hy926 cells were lysed in CHAPS IP lysis buffer and 1000 μg of the clarified protein extract was incubated with either 8 μg anti-eNOS rabbit polyclonal antibody or 8ug isotype control normal rabbit IgG. Immunoprecipitated proteins were subjected to Western blotting analysis with anti-eNOS antibody mouse monoclonal (upper blot) and anti-LSP1 rabbit polyclonal antibody (lower blot). Blue arrows indicate residual signal from immunoglobulin heavy chain of denatured antibody utilized to pull down eNOS. Black arrow denotes additional faint western blot signal that is also expressed in input (whole cell lysate before immunoprecipitation) and is likely representing another isoform of human LSP1. Data are representative of three independent experiments, *n*=3. (**B**) Representative immunoblotting analysis of LSP1 immunoprecipitates in mouse cardiac tissue lysates. Murine heart tissue lysates from wild type and LSP1 KO mice were lysed by cryogrinding in Pierce IP lysis buffer and were incubated overnight with 4 μg anti-LSP1 rabbit polyclonal antibody (Santa Cruz), and immunoprecipitated protein complexes were immunoblotted with mouse-monoclonal anti-eNOS antibody (top), and rabbit polyclonal anti-LSP1 (bottom). Cardiac tissue lysate from LSP1 KO mice was incubated with same amount of antibody and was used as negative control in coimmunoprecipitation experiment. Data are representative of two experiment, *n*=2

To examine specificity of the interaction, we substituted anti-tGFP antibody for non-immune mouse IgG and we probe immunoblot with anti-GAPDH antibody (protein not expected to interact with LSP1). As shown in post-IP lane of Figure 3.7 (*upper panel*), the majority of endothelial eNOS pool was unbound to LSP1-tGFP protein, implying that only minor fraction of eNOS was associating with exogenous LSP1.
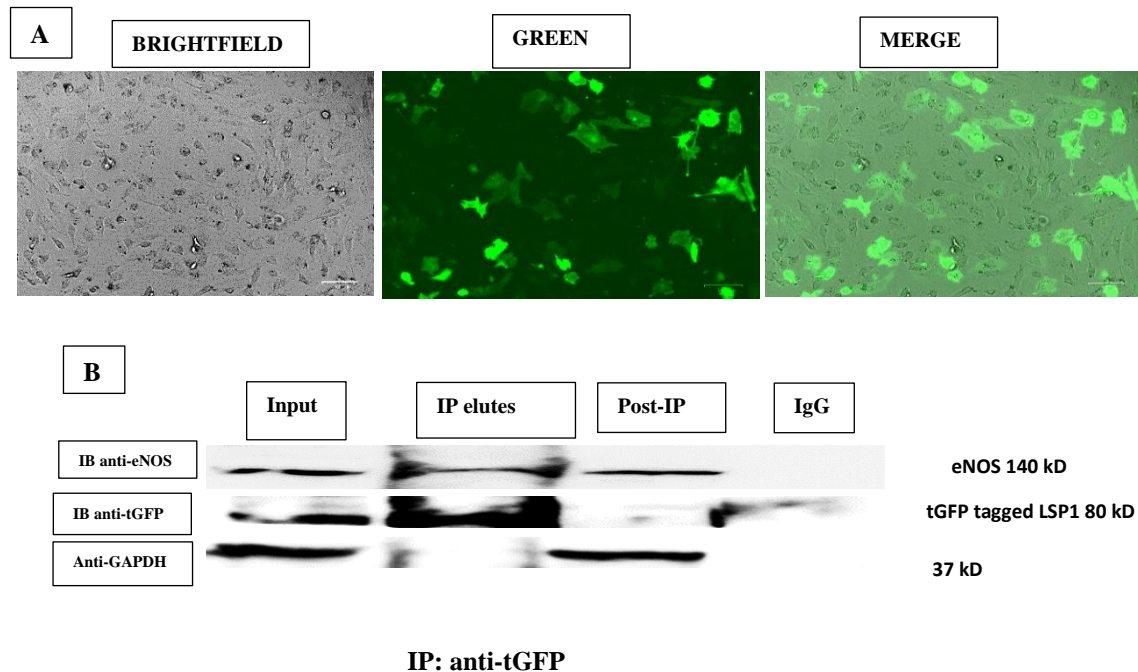
Figure 3.7: Coimmunoprecipitation of LSP1-tGFP and eNOS. **A** Fluorescent microscopic images of LSP1-tGFP expression 48 hours post-transfection with cDNA mammalian expression vector coding for human LSP1 tagged with tGFP at carboxyl terminus. The images were captured by inverted Bio-Rad Zoe fluorescent cell imager and illustrate that 30% of endothelial cells were tGFP positive. **B** Pull down of tGFP-tagged human LSP1 and Coimmunoprecipitation of eNOS. EA.hy926 cells stably transfected with tGFP LSP1 cDNA were lysed in Pierce IP lysis buffer and purified protein extracts were subjected to immunoprecipitation by anti-turbo GFP mouse monoclonal antibody or non-immune mouse IgG. Fractions (input, IP elute, and post-IP supernatant) were resolved by SDS-PAGE electrophoresis and labelled with anti-eNOS mouse monoclonal antibody (top), and anti-turbo GFP antibody mouse monoclonal antibody (middle), and anti-GAPDH mouse monoclonal antibody (bottom) to demonstrate specificity of the interaction. Data are representative of three independent experiments, *n*=3.

To further confirm our hypothesis that human LSP1 and eNOS interact within human endothelial cells, we carried out colocalization study by confocal microscopy. EA.hy926 cells cultured in specialized tissue culture treated chambered glass slides were immunolabelled with anti-eNOS and anti-LSP1 antibodies. In our data, both human LSP1 and eNOS existed within their anticipated subcellular compartments in endothelial cells. As seen in Figure 3.8 (*upper left panel*), eNOS resides predominantly in perinuclear compartment and distinct areas of plasma membrane with scattered weak signal in cytosol, whereas native LSP1 was primarily localized in the nucleus and additional signal emanating from perinuclear structures (Fig 3.8, *upper right panel*). As seen in the merged images of Figure 3.8 (*lower right panel*), human LSP1 colocalized with eNOS in characteristic semilunar perinuclear distribution pattern (yellow). Next, we investigated the distribution and colocalization of exogenous LSP1-tGFP and eNOS. To assess if LSP1-tGFP protein colocalizes with eNOS, stably transfected EA.hy926 cells were solely immunolabelled with mouse monoclonal anti-eNOS antibody. As seen in Figure 3.9 (*top left panel*), LSP1-tGFP resides primarily in nucleus and perinuclear compartment, and in composite images, it colocalizes with eNOS primarily in perinuclear region (*bottom right panel*). Remarkably, both native LSP1 and stably transfected tGFP-LSP1 protein share similar pattern of colocalization with eNOS in human macrovascular endothelial cells. However, native LSP1 gave rise to the strongest colocalization signal, likely because it has the proper conformation structure and posttranslational modifications to bind more avidly to eNOS compared to exogenous protein.

To further support our findings of biochemical interaction of LSP1 and eNOS, we have performed immunoprecipitation of mouse LSP1 from WT cardiac tissue homogenates under non-denaturing conditions using anti-LSP1 rabbit polyclonal antibody and we have successfully identified eNOS in LSP1 immunoprecipitates (Figure 3.6B, *upper panel*). For this coimmunoprecipitation experiment, we have utilized heart tissue lysate from LSP1 KO mice as negative control and subject it to the same CO-IP conditions as WT heart lysates (i.e., same amount of antibody and magnetic beads, etc.). The use of knockout animal as negative control in Coimmunoprecipitation has been recommended in literature (Free, Hazelwood & Sibley, 2009) to verify specificity of IP antibody. Furthermore, as seen in Supplementary Figure 1 (*top panel*), strong eNOS signal was present in WT IP lane but was barely detectable in LSP1 KO IP lane, which suggest that anti-LSP1 rabbit polyclonal antibody (Santa Cruz) is specific and has minimal

cross reactivity with eNOS. Collectively, our data suggest that LSP1 and eNOS interaction occur in the endogenous tissue milieu as well as human endothelial cells.

Taken together, our studies suggest that LSP1 and eNOS associate physically with each other in human macrovascular endothelial cells and mouse cardiovascular tissues.
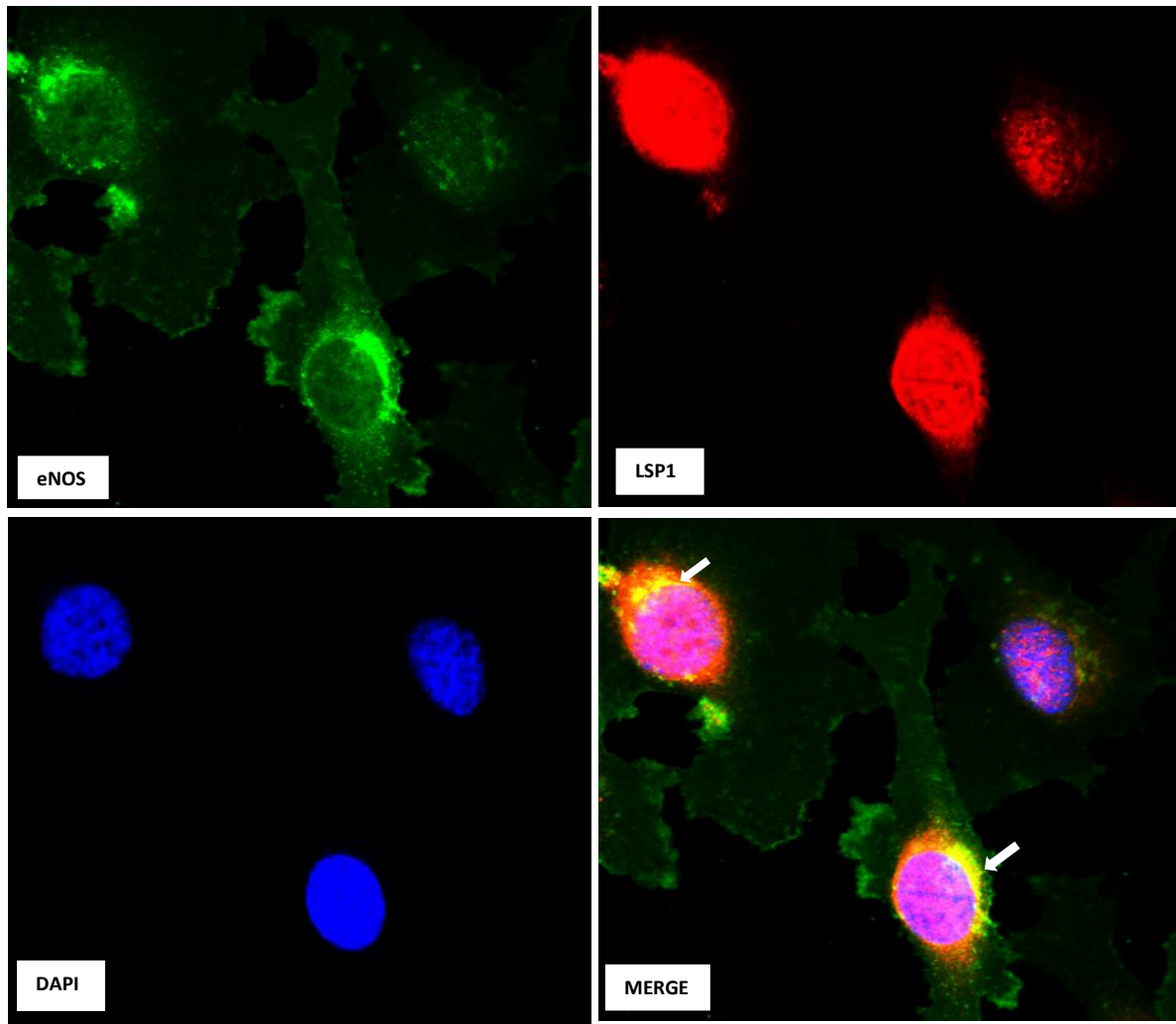
Figure 3.8: Colocalization of native LSP1 and eNOS in human macrovascular endothelial cells. EA. hy926 cells were cultured on Poly D-lysine treated tissue culture glass slides and were fixed, permeabilized, and immunolabelled with mouse monoclonal anti-eNOS (**green**) and rabbit polyclonal LSP1 (**red**) primary antibodies, followed by detection with Alex Fluor 488-labelled goat anti-mouse and Alexa Fluor 647-labelled donkey anti-rabbit secondary fluorescent probes. Note typical localization of eNOS to perinuclear region and distinct plasma membrane pools, with fainter staining in cytosol. Arrows denote significant colocalization of eNOS and LSP1 (**yellow**) in merged image, particularly in perinuclear region. Immunofluorescence images were captured by confocal microscopy using 63X oil lens.
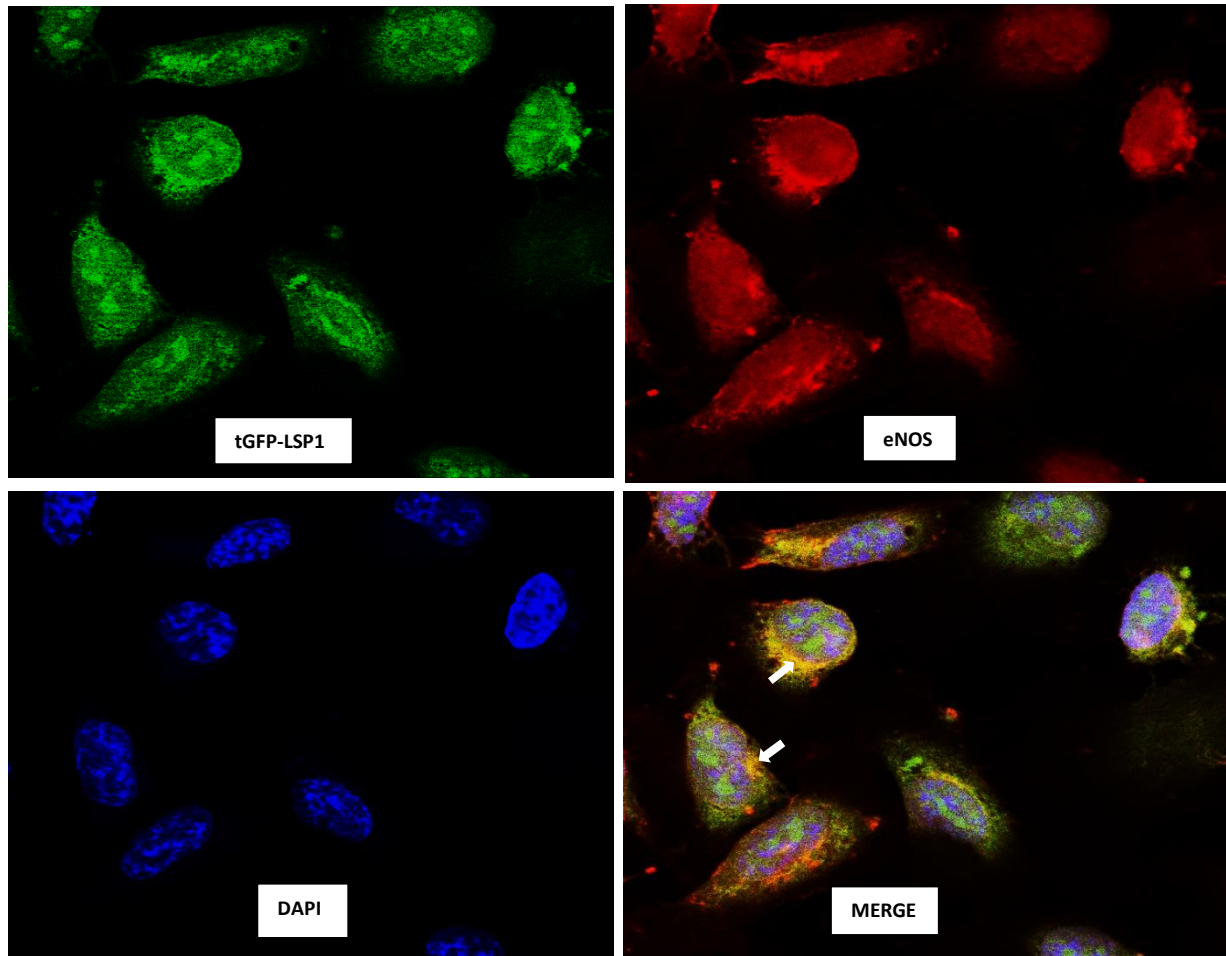
Figure 3.9: Colocalization of exogenous LSP1-tGFP and eNOS in human macrovascular endothelial cells. EA. hy926 Cells stably transfected with LSP1-tGFP cDNA vector were exclusively immunolabelled with mouse monoclonal antibody to eNOS, followed by identification with Alexa Fluor 568-conjugated donkey anti-mouse secondary antibody. Arrows indicate areas of colocalization between two proteins in the merged image. Turbo-GFP tagged human LSP1 colocalizes with eNOS in distinct perinuclear distribution. The pattern of colocalization of exogenous LSP1 is consistent with native LSP1 colocalization with eNOS. The images were visualized under confocal microscopy using 40X oil lens.

3.5. Impaired NO production in LSP1 deficient human endothelial cells.

To assess whether LSP1 knockdown in EA.hy926 cells has an impact on NO generation (measure of eNOS activity) from endothelial cells, we knockdown human LSP1 in EA.hy926 cells and intracellular NO levels were measured by NO-specific and sensitive fluorescent probe (DAF-FM). Since eNOS is calcium/calmodulin-dependent enzyme, we decided to test both basal and thapsigargin stimulated NO release. Thapsigargin is a selective inhibitor of endoplasmic reticulum Ca^{2+}-ATPase pump, eventually leading to depletion of intracellular calcium stores and activation of store-operated calcium channels (capacitative calcium entry) (Putney, 2010). It has been shown that capacitative calcium entry induced by thapsigargin results in sustained activation of eNOS (Lin, Fagan, Li, Shaul, Cooper & Rodman, 2000). Furthermore, thapsigargin has been demonstrated to be the most potent calcium mobilizing agonist for stimulation of nitric oxide production in endothelial cells (Wang et al., 1996). As shown in Figure 3.10, both the basal and thapsigargin-stimulated NO synthesis were significantly lower in LSP1 deficient EA.hy926 cells compared to control endothelial cells transduced with control vector ($p < 0.05$, $p < 0.01$, respectively). However, thapsigargin-stimulated nitric oxide production was more significantly impaired than basal nitric oxide output (more than 4-fold reduction in NO synthesis compared to 2-fold decrease in basal NO output; Fig. 3.10, *lower panels*), suggesting that calcium-dependent activation of eNOS is blunted following knockdown of human LSP1. This could be attributed to the fact that LSP1 is inherently calcium binding protein. In addition, it is most likely that the reduction in basal NO production in LSP1 deficient EA. hy926 cells is secondary to reduced expression of eNOS following knockdown of human LSP1. It appears that there is a correlation between reduction of basal NO synthesis and the observed downregulation of eNOS following depletion of human LSP1(Fig 3.1A *upper panel*, and Fig 3.10B). Since we have previously demonstrated that LSP1 and eNOS coexist as protein complex in human endothelial cells, it is also likely that LSP1 directly modulates enzymatic activity or phosphorylation status of eNOS and that deficiency of LSP1 contribute to impairment of nitric oxide release.
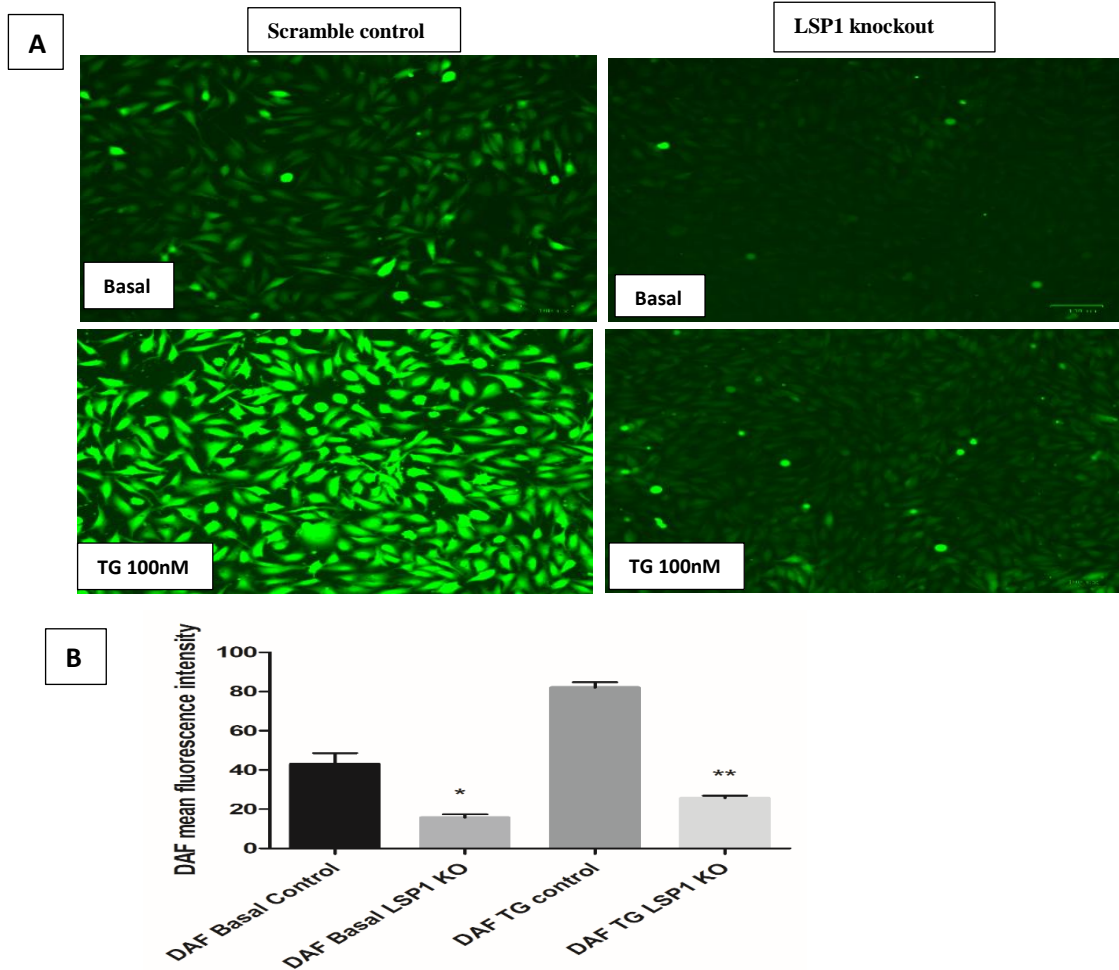
Figure 3.10: The effect of LSP1 knockout on basal and agonist-induced NO production in human macrovascular endothelial cells. Seven days post lentiviral transduction with scramble control or target 3 sgRNA CRISPR/Cas9 against human LSP1, DAF-FM loaded EA. hy926 cells were stimulated with thapsigargin (100 nM) for 1 hour. Thapsigargin is a potent calcium-mobilizing agonist for nitric oxide release in endothelial cells. (**A**) Fluorometric quantification of basal and thapsigargin-stimulated NO production in scramble control and LSP1 KO groups. (**B**) analysis of mean fluorescence intensity of data shown in (a). Data are presented as mean ± SEM of two independent experiments $n=2$. * denotes $p < 0.05$, ** $p < 0.01$ by t test, comparing each knockout group to the corresponding scramble control group. Scale bar = 100 μm.

3.6. LSP1 deficiency leads to increased expression of eNOS, eNOS uncoupling and enhanced susceptibility of eNOS to proteolytic degradation in mice.

Give the complex nature of NO signalling in vivo, we sought to further examine effect of LSP1 gene disruption on eNOS expression. First, we investigated whether endothelial dysfunction in LSP1 KO mice is associated with eNOS uncoupling, and we performed LT-SDS PAGE to study the coupling status of eNOS in vivo. As shown in Figure 3.11A, the formation of eNOS dimer was significantly inhibited in isolated aorta blood vessels of LSP1 KO mice, associated with prominent appearance of eNOS monomer band, whereas eNOS from age-matched WT mice aorta showed prominent eNOS dimer band with barely detectable eNOS monomer. eNOS dimer/monomer ratio (Fig. 3.11B) was significantly higher in WT compared to LSP1 KO mice (p value<0.05, $n=8$). These data suggest that adequate supply of LSP1 is crucial for maintaining dimerization status of eNOS. Next, we have performed western blotting on aorta and mesenteric arteries tissue homogenates from age-matched WT and LSP1 KO mice. As shown in Figure 3.12A and B (*upper panels*), the expression of eNOS was significantly greater in aortae and mesenteric arteries of LSP1 KO mice compared to their corresponding age-matched WT mice ($p< 0.001$, and $p < 0.01$, respectively). eNOS protein abundance increased by more than 7-fold in aorta tissues (Figure 3.12C) and more than 4-fold for mesenteric arteries (Figure. 3.12D). Besides eNOS dysregulated expression and uncoupling, we have observed potential eNOS cleavage fragments in Aorta and mesenteric arteries tissue lysates from LSP1 KO mice at molecular weight of 100kD (Figure 3.12A, *upper panel*), and at molecular weight of 75kD, and 60 kD (Figure 3.13 A and B, *upper panel*).
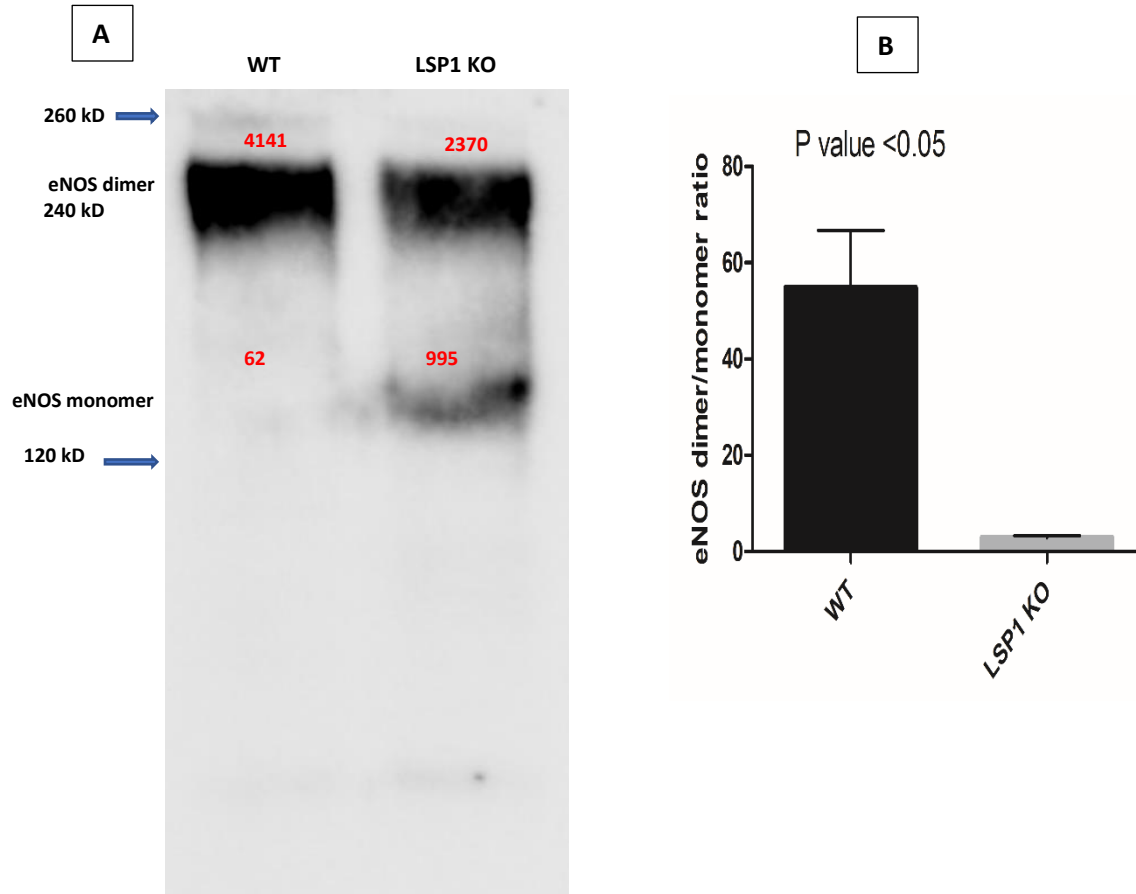
Figure 3.11: The effect of LSP1 knockout on the coupling status of endothelial nitric oxide synthase. (**A**) Representative immunoblotting of aortic eNOS dimer and monomer bands. Aorta blood vessels from WT and LSP1 Knockout mice were homogenized in Pierce immunoprecipitation lysis buffer, and total protein extracts (40μg/lane) were subjected to non-denaturing (no-boiling) non-reducing low temperature SDS PAGE electrophoresis (LT-PAGE) to maintain dimerization status of eNOS. Dimer and monomer forms of eNOS were detected with mouse monoclonal anti-eNOS antibody (BD-bioscience). Numbers highlighted in red denote densitometry measurements of eNOS dimer and monomer bands using Image Lab Software (Bio-Rad). (**B**) Graph demonstrating quantification analysis of eNOS dimer-to-monomer ratio, *p value*=0.04 by *t*-test. The experiment was repeated two times with comparable results, *n*=12 mice, 6 mice for each WT and LSP1 KO experimental groups for two experiments combined (3 mice for each experiment).
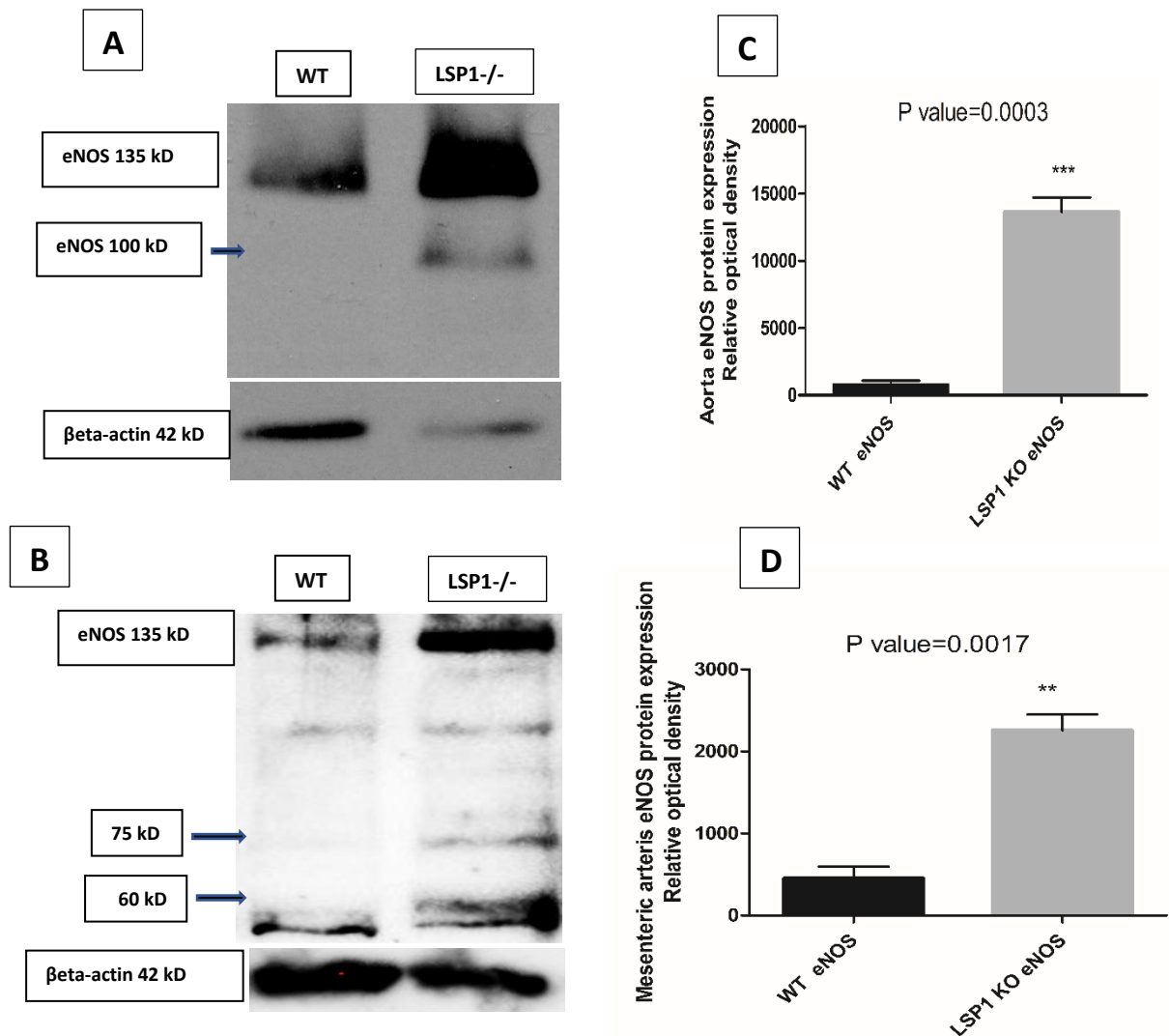
Figure 3.12: eNOS expression in vascular tissues from WT and LSP knockout mice. Representative western blotting of eNOS protein expression in aorta (**A**) and mesenteric arteries (**B**) from 12 to 16 weeks aged WT and LSP1 KO mice. (**C** and **D**) Graph data illustrate optical densities of eNOS bands in Aorta (top) and mesenteric arteries (bottom), respectively. The results are displayed as mean ± SEM of three independent experiments, *n*=12 mice for each WT&LSP1 KO experimental groups for 3 experiments combined (4 mice for each experiment). ** indicate p< 0.01 and *** p < 0.001 between wild type and LSP1 knockout groups.
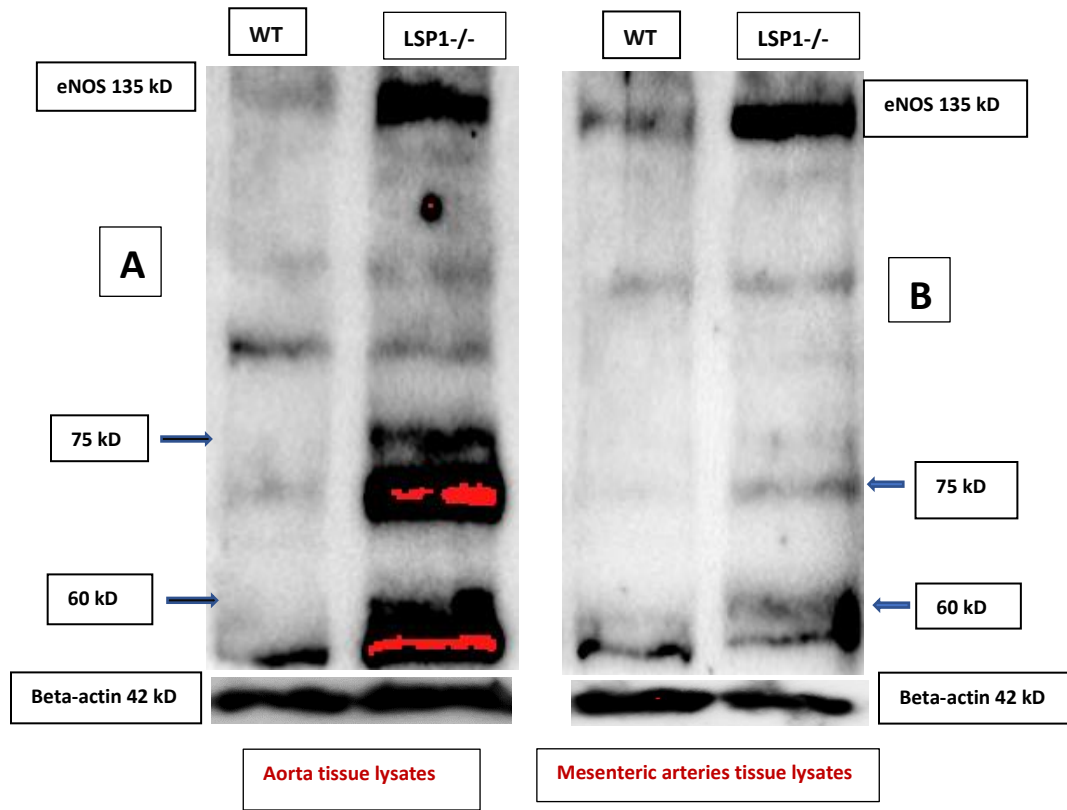
Figure 3.13: eNOS cleavage in vascular tissues of LSP1 KO mice. (**A**) Aorta and (**B**) mesenteric arteries tissue lysates from WT and LSP1 KO mice were lysed by cryogrinding in CHAPS lysis buffer, and 30 μg of protein per lane from each sample were resolved by SDS-PAGE gels. The immunoblots were probed with anti-eNOS rabbit polyclonal antibody (top) and anti- βeta actin mouse monoclonal antibody (bottom). The rabbit polyclonal anti-eNOS antibody has revealed 75 and 60kD immunoreactive bands (particularly in Aorta samples) which are likely cleavage products of eNOS. eNOS cleavage products are indicated by blue arrows. Data are representative of two experiments, *n=2*.

4. DISCUSSION

Although endothelial LSP1 has been previously investigated in the context of inflammation and endothelial permeability, there has been scientific gap regarding the role of LSP1 in endothelial dysfunction. In this study, we hypothesized that LSP1 has an important role in endothelial function via regulation of expression and function of eNOS, and we have investigated our hypothesis utilizing an in-vitro cell culture model and LSP1 knockout mice. Data presented in this thesis provide first evidence suggesting that LSP1 plays a crucial role in regulation of eNOS expression and function. LSP1 deficiency promotes endothelial dysfunction in human endothelial cells and induces hypertension in animal model (LSP1 null mice). Downregulation of eNOS protein expression was observed following transient knockdown or knockout of human LSP1 via CRISPR/Cas9 system delivered by either plasmid transfection (Figure 3.1A) or Lentivirus transduction (Figures 3.2A, 3.3A), and eNOS expression levels appears to parallel the changes in LSP1 expression levels. Moreover, human LSP1 loss-of-function was associated with a significant reduction is the abundance of eNOS mRNA by more than 70% (Figure 3.4A). This most likely reflects inhibited transcriptional rate of eNOS gene. There seems to be a good correlation between changes in eNOS mRNA and the observed downregulation at eNOS protein level (Figures 3.1A and 3.4A). In addition, LSP1 gain-of-function via adenovirus transduction was associated with marked overexpression of eNOS (Figure 3.5A, *upper panel*) at the protein level. Furthermore, we report that LSP1 physically interact with eNOS under basal physiological conditions (Figures 3.6, 3.7, 3.8 and 3.9). Collectively, these data indicate that LSP1 is novel regulator of eNOS expression and function.

Although actin and actin-binding proteins were initially considered as structural proteins in many previous studies, there have been recent reports suggesting otherwise, and advocating that actin and actin-binding proteins may act as regulators of transcription (Gettemans, Van Impe, Delanote, Hubert, Vandekerckhove & De Corte, 2005; Louvet & Percipalle, 2009; Zheng, Han, Bernier & Wen, 2009). Furthermore, recent study has identified nuclear βeta-actin as a novel transcriptional modulator of eNOS expression (Ou et al., 2005), providing evidence that transcriptional activity of actin and actin-binding proteins exist within endothelial cells, and that

this signalling pathway is involved in regulation of basal eNOS expression. Because LSP1 is essentially an actin-binding protein (Jongstra-Bilen, Janmey, Hartwig, Galea & Jongstra, 1992; Li, Guerrero & Howard, 1995; Zhang, Li & Howard, 2000) and since endothelial LSP1 is primarily localized in the nucleus (Liu et al., 2005), it is likely that LSP1 modulates eNOS gene expression at the transcriptional level. To explore our hypothesis that LSP1 modulates expression of eNOS at transcriptional level, we have performed quantitative real-time PCR and immunoblotting to quantify gene expression of eNOS in cultured human endothelial cells following depletion of LSP1 by CRISPR/Cas9 deletion machinery, and we have observed significant downregulation of eNOS at mRNA (Figure 3.4A) and protein levels (Figure 3.1A, *upper panel*). Because actin cytoskeleton has been demonstrated as an important posttranscriptional mechanism that regulates half-life of eNOS mRNA transcripts in endothelial cells (Searles, 2006; Searles, Ide, Davis, Cai & Weber, 2004), we investigated the possibility that the reduction in eNOS mRNA abundance observed after depletion of human LSP1 via CRISPR/Ca9 was occurring due to decreased mRNA stability of eNOS. Our results (Figure 3.4C) indicate that following treatment of control and LSP1 depleted endothelial cells with actinomycin D (transcriptional inhibitor) for various time points, there was no significant difference in mRNA decay of eNOS between two groups, supporting our hypothesis that downregulation of eNOS mRNA levels following silencing of LSP1 appears to be secondary to lowered transcriptional rate of eNOS gene. The decrease in transcriptional activity of eNOS gene was anticipated given the fact that LSP1 silencing in endothelial cells by siRNA has been shown to reduce the expression of transcription factor GATA2 (Hossain et al., 2015). GATA2 transcription factor is known to bind to eNOS promoter region and influences the basal transcriptional rate of eNOS gene (Zhang, Min & Sessa, 1995), therefore it is plausible to conclude that signalling pathway involving nuclear LSP1 and downstream GATA2 is probably involved in transcriptional regulation of eNOS. This conclusion was further supported by observation that adenoviral mediated overexpression of human LSP1 gene upregulates expression of eNOS (Figure 5A, *upper panel*).

EA.hy926 cells are immortalized HUVEC derived from fusion of primary HUVEC and lung carcinoma cell line A549 (Edgell, McDonald & Graham, 1983). We have chosen EA.hy926 cells as an in-vitro model to study role of human LSP1 in endothelial dysfunction because they have robust growth characteristics (Edgell, Curiel, Hu & Marr, 1998) and maintain differentiated

endothelial cell phenotype (Rieber, Marr, Comer & Edgell, 1993), conserve many features and markers from primary HUVEC (Ahn, Pan, Beningo & Hupe, 1995; Bouis, Hospers, Meijer, Molema & Mulder, 2001), and are presently the best characterized macrovascular endothelial cell (Bouis, Hospers, Meijer, Molema & Mulder, 2001). We opted to use adenoviruses for overexpression of human LSP1 because EA.hy926 cells are difficult to transfect cell line (Edgell, Curiel, Hu & Marr, 1998; Teifel, Heine, Milbredt & Friedl, 1997), and also because of high efficiency of utilizing adenoviruses as gene delivery method in endothelial cells (Edgell, Curiel, Hu & Marr, 1998). Furthermore, adenoviruses are considered the most efficient gene delivery methods because they possess many advantages over other gene transfer vectors (Benihoud, Yeh & Perricaudet, 1999; Crystal, 2014; Lee et al., 2017), for instance, transduction of both dividing and quiescent cells, the ability to package large insert up to 7.5 kb, adenoviral DNA remains Episomal and does not integrate into host genome and therefore there is a minimal risk of insertional mutagenesis compared to lentiviral vectors, amplification into very high titers up to 1×10^{13}, and most importantly highest transduction efficiency and strongest transgene expression.

Endothelial nitric oxide synthase is a large molecule that is subject to multiple mechanisms of regulation at transcriptional, post-transcriptional and post-translational levels, including protein-protein interactions (Govers & Rabelink, 2001; Searles, 2006). Several proteins have been shown in the literature to associate with eNOS and influence eNOS function in positive or negative manner. Among these proteins, HSP90 and calpain directly interact with and activate eNOS (Averna et al., 2008), whereas, caveolin-1 strongly binds to and inhibit eNOS activity (Ju, Zou, Venema & Venema, 1997). We report in this study that the two proteins LSP1 and eNOS associate with each other under basal physiological conditions (Figures 3.6 to 3.9). Furthermore, our findings of impaired nitric oxide production from endothelial cells after depletion of human LSP1 (Figure 3.10A), and observation that LSP1 knockout mice have hypertensive phenotype and impaired nitric oxide-dependent vasodilation (unpublished data) provide evidence that the interaction between two proteins is not solely physical but is also relevant to regulation of eNOS function. Our colocalization data using confocal microscopy denote that interaction occurs predominantly in semilunar perinuclear distribution in human endothelial cells (Figures 3.8, *lower right panel*). Interestingly, multiple intracellular proteins are found in literature to colocalize with eNOS in endothelial cells in similar perinuclear pattern (Alvira et al., 2012; Cao et al., 2001). Our

confocal fluorescent microscopy data (Figure 3.8, *upper left panel*) indicate that eNOS is primarily localized in perinuclear region and in discrete areas of plasma membrane with fainter staining pattern in the cytosol, and are consistent with previous studies (Fulton et al., 2002) which establish the existence of two pools of active eNOS (in perinuclear and plasma membrane locations). Furthermore, LSP1 colocalization with eNOS specifically in perinuclear region and not in plasma membrane, may have significant functional implications for the activity of the enzyme. Studies have demonstrated that perinuclear region is the most efficient location for phosphorylation of eNOS and nitric oxide generation from active enzyme (Church & Fulton, 2006; Fulton et al., 2004; Fulton et al., 2002; Sessa et al., 1995) in endothelial cells. The preferential localization of eNOS to perinuclear region and plasma membrane is conferred by means of certain post-translational modifications, specifically N-terminal myristoylation and palmitoylation, (Church & Fulton, 2006). Acylation-deficient eNOS is confined to cytosol and releases less nitric oxide (Church & Fulton, 2006; Rafikov et al., 2011). Other study (Sanchez, Savalia, Duran, Lal, Boric & Duran, 2006) claims that selective localization of eNOS to perinuclear region and cytosol will yield nitric oxide with specific physiological actions. Furthermore, nitric oxide output from perinuclear eNOS is implicated in vasodilation response, whereas nitric oxide released from cytosolic enzyme is involved predominantly in hyperpermeability changes. In this study, no specific fluorescent signal was observed in negative control slides in which endothelial cells were incubated with secondary fluorescent probes only without primary anti-LSP1 and anti-eNOS antibodies. Although we have also observed colocalization of exogenous turbo-GFP tagged LSP1 and eNOS in human endothelial cells (Figure 3.9, *lower right panel*), and in similar perinuclear distribution, it appears that native LSP1 is more strongly colocalized with eNOS than exogenous LSP1. Furthermore, it is likely that native LSP1 has more binding affinity than overexpressed LSP1-tGFP protein. Since we have utilized coimmunoprecipitation and confocal colocalization methodologies to reveal LSP1 interaction with eNOS, we can not ascertain that interaction we have observed between two proteins is direct as this assumption would require other protein-protein interaction detection methods such as yeast two-hybrid and in-vitro binding assays. However, there are numerous limitations and drawbacks of those techniques (Bell, Engleka, Malik & Strickler, 2013; Berggard, Linse & James, 2007; Brymora, Valova & Robinson, 2004), for instance, the lack of post-translational modification and improper protein conformation in yeast-two hybrid system and in the in-vitro binding assay (pull down assay) if fusion protein was purified from bacteria.

Furthermore, commonly used in-vitro binding assay is GST-pull down assay, however, the solubility of GST-fusion protein and intrinsic propensity of GST to dimerize (Bell, Engleka, Malik & Strickler, 2013) are additional challenges when utilizing this method to verify direct protein-protein interactions.

Human eNOS gene is located on the long arm of chromosome 7 and is composed of 26 exons corresponding to 21kb stretch (Marsden et al., 1993). Several polymorphisms in eNOS gene have been documented in the literature and are linked to the severity of cardiovascular disease (Chang et al., 2003; Colombo et al., 2003; Esparza-Garcia et al., 2015; Rossi et al., 2003). The most extensively studied mutation to affect coding sequence of eNOS gene is Glu298Asp variant resulting in substitution of glutamate for aspartate at position 298. Glu298Asp polymorphism in eNOS gene has been correlated with primary hypertension in number of studies (Benjafield & Morris, 2000; Hingorani, 2003; Kishimoto et al., 2004; Li et al., 2011). Endothelial nitric oxide synthase protein carrying aspartate but not glutamate at position 298 is susceptible to proteolytic degradation, resulting in generation of C-terminal 100kD and N-terminal 35kD fragments (Tesauro, Thompson, Rogliani, Qi, Chaudhary & Moss, 2000). More recent study (Fairchild, Fulton, Fontana, Gratton, McCabe & Sessa, 2001) argues that 100kD cleavage fragment of eNOS could indeed be the result of in-vitro sample preparation under acidic conditions favored by frequent use of lammeli buffer for Western blotting applications. Furthermore, researchers claim that wild type eNOS (lacking Glu298Asp mutation) may also generate 100kd degradation fragment under prolonged acidic incubation conditions. However, researchers in the study have identified after preliminary functional analysis that this 100kD fragment of eNOS is indeed the active reductase domain of eNOS and that it could generate superoxide free radical species if it would have been existing in vivo. Moreover, researchers did not rule out the possibility that 100kD eNOS product could be initiated in vivo by unrevealed proteolytic process. In our data, it remains unclear why we observed 100kD eNOS fragment in cardiovascular tissue lysates from LSP1 knockout mice (Figure 3.12A, *upper panel*). Although we have also seen 100kD eNOS band in wild type tissues, it is much weaker in density compared to the band observed in LSP1 knockout samples. Furthermore, the significantly higher expression of 100kD eNOS band in tissues of LSP1 knockout mice may imply that LSP1 is involved in undisclosed proteolytic pathway, and that the existence of LSP1 confers protection to eNOS against some unknown proteases. Remarkably, our

observations indicate that the appearance of 100kd eNOS degradation product occurs almost exclusively after treatment of protein samples with reducing agent Dithiothreitol (DTT), suggesting that 100kD eNOS product might be the result of in-vitro cleavage process. DTT might be activating some unknown cysteine dependent proteases in vitro, resulting in proteolytic degradation of eNOS. Intriguingly, DTT reducing agent has been shown in number of studies to activate (Mallorqui-Fernandez et al., 2008; Shoshan-Barmatz, Weil, Meyer, Varsanyi & Heilmeyer, 1994; Zeng, Dunlop, Rodgers & Davies, 2006) and stabilize (Croall & DeMartino, 1983) the activity of some calcium-dependent cysteine proteases. However, it is not entirely clear why particularly eNOS from LSP1 knockout mice, but not WT mice, is more susceptible to proteolytic degradation. Remarkably, the 100kD eNOS degradation fragment is detected in our data after probing of immunoblots with mouse monoclonal antibody raised against C-terminus of human eNOS, therefore, supporting the identity of 100 kd protein as eNOS cleavage product, and suggesting that it is most likely derived from C-terminus of eNOS protein, Furthermore, our results are consistent with previous studies (Fairchild, Fulton, Fontana, Gratton, McCabe & Sessa, 2001; Tesauro, Thompson, Rogliani, Qi, Chaudhary & Moss, 2000) which reveal that 100 kD cleavage eNOS product is derived from C-terminus of eNOS. Apart from 100kD cleavage eNOS protein, we have also observed additional protein bands at the molecular weight of 75 and 60 kD in immunoblots from cardiovascular tissues of LSP1 knockout mice (3.13A and B, *upper panels*) that might represent potential eNOS cleavage fragments. Those bands were strongly reactive with rabbit polyclonal anti-eNOS antibody. Intriguingly, one investigative work has shown that exposure of eNOS to superoxide radical species result in cleavage of the enzyme into 75kD and 60kD fragments (Black, Ross, Levic & Hallmark, 1999).

In an attempt to discern the cause of hypertension in LSP1 knockout mice, we examined the expression of endothelial nitric oxide synthase in cardiovascular tissues of wild type and LSP1 null mice aged 12 to 16 weeks. LSP1 knockout mice showed marked upregulation of eNOS expression in aortae, and mesenteric arteries (Figure 3.12A and B). Our results are consistent with previous studies which demonstrate that endothelial dysfunction in hypertension is mostly associated with increased rather than decreased eNOS expression (Li, Wallerath, Munzel & Forstermann, 2002; Llorens, Salazar & Nava, 2005; Vaziri, Ni & Oveisi, 1998). Increased expression and activity of eNOS have been reported in cardiac tissues of spontaneously hypertensive rats as compensatory

effort against high arterial blood pressure (Nava, Noll & Luscher, 1995). Because endothelial dysfunction is associated with diminished nitric oxide availability, upregulation of eNOS has been elucidated as the consequence of loss of normal negative feedback inhibition by NO (Vaziri & Wang, 1999). However, more recent investigations have attributed the upregulation of eNOS to oxidative stress associated with endothelial dysfunction (Li, Wallerath, Munzel & Forstermann, 2002; Vaziri, Ding & Ni, 2001; Zhen, Lu, Wang, Vaziri & Zhou, 2008). It has been suggested that ROS-induced upregulation of eNOS is at least partially mediated by loss of negative feedback loop regulation of eNOS by nitric oxide (Zhen, Lu, Wang, Vaziri & Zhou, 2008). It should be noted that reactive oxygen species such as hydrogen peroxide could activate transcription of eNOS gene and prolong the half-life of eNOS mRNA transcripts (Drummond, Cai, Davis, Ramasamy & Harrison, 2000). In addition, hydrogen peroxide can also induce expression of eNOS via Calcium/calmodulin-dependent protein kinase II (CaM kinase II) pathway (Cai, Davis, Drummond & Harrison, 2001). However, the compensatory upregulation of eNOS in setting of endothelial dysfunction is often considered as futile mechanism, because upregulated eNOS is most likely uncoupled malfunctioning enzyme that will generate superoxide rather than nitric oxide, therefore aggravating vascular oxidative stress and causing more severe scavenging of nitric oxide (Forstermann & Li, 2011; Li, Wallerath, Munzel & Forstermann, 2002; Zhao, Vanhoutte & Leung, 2015). It is likely that upregulated eNOS in vascular tissues of LSP1 knockout mice is ramification of reduction in nitric oxide bioavailability. This conclusion is supported by observation of impaired nitric oxide-dependent vasodilation in mesenteric resistance arteries of LSP1 knockout mice (data not shown here), and our data of impaired basal and agonist stimulated-NO production in human macrovascular endothelial cells following silencing of LSP1 gene (Figure 3.10A). However, we can not rule out possibility that there is vascular oxidative stress in LSP1 KO mice, and that enhanced generation of ROS species like hydrogen peroxide is causing upregulation of eNOS protein abundance by mechanisms outlined above. In addition, we have observed uncoupling of eNOS in isolated aorta blood vessels from LSP1 KO mice (Figure 3.11A). eNOS uncoupling has been reported in several animal models of hypertension, such as, spontaneously hypertensive rats (Bhatt, Lokhandwala & Banday, 2011), angiotensin II-dependent hypertension (Mollnau et al., 2002), and in deoxycorticosterone acetate (DOCA)-salt hypertension (Landmesser et al., 2003). eNOS uncoupling has also been noticed in patients with essential hypertension (Higashi et al., 2002). It is likely that eNOS uncoupling plays significant role in the pathogenesis of hypertension

in LSP1 KO mice since the reversal of eNOS uncoupling has proven to be efficient strategy to lower blood pressure in hypertensive states (Forstermann & Li, 2011; Forstermann & Munzel, 2006; Li et al., 2006).

Data from the current study suggest that LSP1 exerts regulatory effect on eNOS at two distinct transcriptional and posttranslational phases. CRISPR/Cas9-induced knockout of human LSP1 and adenovirus-mediated overexpression of human LSP1 have revealed the positive upregulatory role of LSP1 in regulation of eNOS transcription. The nuclear localization of LSP1 in endothelial cells favors its role as transcription factor or transcriptional activator. At posttranslational levels, LSP1 exercises its effect on eNOS at multiple aspects. First, LSP1 modulate enzymatic activity of eNOS as manifested by marked reduction in basal and agonist-stimulated Nitric oxide output following knockdown of human LSP1. In addition, adequate supply of LSP1 might be crucial to preserve dimerization status of eNOS in vivo. We believe that the modulation of Nitric oxide output from active eNOS as well as role of LSP1 in maintaining proper coupling status of eNOS are probably mediated by biochemical interaction between two proteins (eNOS and LSP1) in endothelial cells. Furthermore, it is intriguing to find that eNOS in LSP1 KO mice is more susceptible to proteolysis, proposing that LSP1 might be essential in conferring protection to eNOS against proteolytic cleavage. It is remarkable how could LSP1 as a cytoskeleton-associated and calcium-binding protein influence eNOS at several complex levels of regulation.

It should be noted that while eNOS expression profiles from LSP1 KO mice and human endothelial cells in this study are different, they do not necessarily contradict each other. In fact, eNOS expression data from the two models might represent different stages in the natural course of endothelial dysfunction and hypertension. Downregulation of eNOS expression shortly following knockdown of human LSP1 might represent initiation phase of endothelial dysfunction where LSP1 function is suddenly lost and eNOS expression is markedly affected, whereas upregulation of eNOS expression in LSP1 KO mice probably reflect compensatory mechanism to restore normal nitric oxide availability, and most likely represent the maintenance phase of hypertension. It is interesting to note that eNOS expression pattern changes during different stages of development of hypertension in animal models (Li, Wallerath, Munzel & Forstermann, 2002). Further studies will likely be required to explore eNOS expression in young LSP1 KO mice in prehypertensive stage (i.e., before onset of hypertension). One possible reason for the opposite

effects of LSP-1 knockout *in vitro* in cultured cells versus *in vivo* in whole vessels could also because *in vivo* in the whole vessel there are different types of cells such as endothelial, smooth muscle and adventitial cells influencing each other, and they might have different expressions or no expression of either eNOS or LSP-1.

One interesting observation during our experiments on overexpression of LSP-1 in endothelial cells was a change in morphology. The endothelial cells were more rounded compared to control endothelial cells which had a more stellate appearance. We did not pursue this further, but it can be investigated if it alters functions of the cells with overexpressed LSP-1.

5. CONCLUSIONS

1-Knockout of human LSP1 using CRISPR/Cas9 technology in endothelial cells downregulates eNOS expression (at mRNA and protein levels) and impairs eNOS function (i.e., nitric oxide production).

2- Human LSP1 gain-of-function via adenovirus-mediated overexpression is associated with enhanced expression of eNOS in human endothelial cells.

3- Physical and functional interaction between LSP1 and eNOS.

4-LSP1 deficiency in mice had dysregulated eNOS expression in mouse cardiovascular tissues.

5-eNOS from LSP1 KO mice is likely susceptible to proteolytic degradation by unrevealed mechanisms**.**

6. FUTURE PERSPECTIVES

It appears that endothelial LSP1 in the nucleus has an important role in regulation of transcriptional processes and is likely transcription factor. However, future work will be required to elucidate detailed molecular mechanisms of how endothelial LSP1 affects GATA2 transcription factor and eNOS expression. Oligonucleotide pull-down assay, Electrophoretic mobility shift assay (EMSA), chromatin immunoprecipitation will be useful strategies to study the role of endothelial nuclear LSP1 in regulation of transcription. Alternatively, overexpression of LSP1 in human endothelial cells by adenoviruses followed by transfection of luciferase gene-eNOS promoter vector would be efficient approach to study role of LSP1 in regulation of eNOS transcription. In addition, more investigative work is needed to reveal the mechanism of increased susceptibility of eNOS from LSP1 KO mice to proteolytic degradation and whether LSP1 is involved in some proteolytic signalling pathways. Apart from the role of LSP1 in regulation of eNOS, more studies will be required to determine mechanisms of eNOS cleavage and characterize the role of eNOS cleavage fragments (100kD, 75kD, and 60kD) in endothelial dysfunction of hypertension and other cardiovascular diseases.

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