Supplemental Document to:

Current siRNA Targets in Atherosclerosis and Aortic Aneurysm

Leena Pradhan-Nabzdyk, Chenyu Huang, Frank W. LoGerfo, and Christoph S. Nabzdyk

Division of Vascular and Endovascular Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, USA.

4. Immune Mediators

4.1 Connection to IKK and SAPK/JNK (CIKS)
CIKS is an upstream regulator of pro-inflammatory molecules, NF-κB and activated protein-1 (AP-1) involved in inflammation modulation in response to injury.
ATH: CIKS silencing reversed high glucose (HG)-induced endothelial-monocyte adhesion and inhibition of EC migration, and inhibited HG-induced inhibitor of NF-κB kinase subunit beta (IKKbeta) and Jun N-terminal kinase (JNK) phosphorylation and nuclear translocation of p65 and c-Jun. It further inhibited NF-κB- and AP-1-dependent expression of pro-inflammatory cytokine, chemokine, and adhesion molecules (Venkatesan *et al.*, 2013).

4.2 NACHT, LRR and PYD domains-containing protein 3 (NALP3)
Nalp3 is part of the NOD-like receptor (NLR) family and is involved in the regulation of inflammation and apoptosis.
ATH: Inflammasome activation has been linked to atherosclerosis. The inflammasome complex including Nalp3, apoptosis associated speck-like protein and caspase1 was upregulated in calcifying VSMC, which led to higher IL-1beta production. Nalp3 silencing mitigated VSMC calcification and IL-1beta production (Wen *et al.*, 2013).

4.3 Interferon regulatory factor (IRF-1)
IRF-1 is a member of the IRF protein family that regulates interferon transcription (Paun & Pitha, 2007).
ATH: Low shear stress and triglyceride-rich lipoproteins (TGRL) enhanced TNF-α-induced vascular cell adhesion molecule-1 (VCAM-1) expression in EC via an IRF-1-dependent mechanism. This effect could be abolished through IRF-1-silencing (Deverse *et al.*, 2013).

4.4 Lipoprotein-associated phospholipases A2 alpha (Lp-PLA(2)alpha)
Lp-PLA(2)alpha is involved in the generation of eicosanoids, potent mediators of inflammation.
ATH: Lentiviral Lp-PLA2 siRNA administered either directly into carotid plaques or systemically reduced pro-inflammatory cytokines whereas anti-inflammatory cytokines were markedly increased in the plasma of mice. The same study showed that local transfection enhanced plaque stability compared to systemic administration (Zhang *et al.*, 2013).

4.5 Cluster of differentiation 40 (CD40)
CD40 is a costimulatory protein expressed on antigen presenting cells and believed to be involved in atherosclerotic plaque pathology.
ATH: Lentivirus mediated CD40 silencing in murine carotid artery plaques decreased plaque progression, lipid content, and intima/media ratios. Additionally, proinflammatory cytokines, chemokines and matrix metalloproteinases were downregulated (Wang *et al.*, 2013).

4.6 Poly(ADP-ribose) polymerase 1 (PARP-1)
The nuclear enzyme PARP-1 is associated with the inflammatory response during atherosclerosis.
ATH: Low shear stress (LSS) increased PARP-1 expression in EC through MEK/ERK. PARP-1 silencing

decreased LSS-induced ICAM-1 upregulation by decreasing nuclear translocation and activity of NF-κB (Qin *et al.*, 2013).

4.7 Interferon-gamma-inducible protein (IP-10) or C-X-C motif chemokine 10 (CXCL10)
IP-10 is secreted by several cells including fibroblasts, macrophages and endothelial cells in response to interferon gamma (Luster *et al.*, 1985). IP-10 is known to regulate leukocyte chemotaxis and to inhibit angiogenesis through inhibition of bone marrow colony forming unit formation (Angiolillo *et al.*, 1995).
ATH: IP-10 is overexpressed in arteriosclerotic lesions and believed to play a role in atherogenesis. IP-10 silencing reduced intima-to-media ratio in a rabbit carotid artery model of atherosclerosis injury (Zuojun *et al.*, 2012).

4.8 F11 Receptor (F11R)
F11R is a cell adhesion protein present constitutively on the membrane surface of circulating platelets and within tight junctions of EC.
ATH: F11R silencing in EC inhibited TNF-α and/or interferon (IFN)-gamma-induced upregulation of F11R mRNA and blocked the adhesion of human platelets to inflamed ECs (Azari *et al.*, 2011). In cytokine activated VSMC, silencing of F11R blocked VSMC proliferation and migration (Azari *et al.*, 2010).

5. Lipid Metabolism

5.1 Siglec-1
Siglec-1 is a member of the sialic acid-binding immunoglobulin-type lectins, which are cell surface proteins involved in cell adhesion and other cellular functions.
ATH: Siglec-1 is expressed on circulating monocytes as well as macrophages in atherosclerotic patients. Ox-LDL up-regulated Siglec-1 expression in macrophages. Silencing of Siglec-1 attenuated ox-LDL uptake into macrophages (Xiong *et al.*, 2013).

5.2 CD36
The cell surface receptor CD36 is a scavenger receptor for cellular ox-LDL uptake and is involved in macrophage to foam cell transformation.
ATH: In macrophages nicotine synergized with ox-LDL to increase expression of CD36, TNF-α, MCP-1, IL-6, and CXCL9. All of these effects were prevented by CD36 silencing (Zhou *et al.*, 2013).

5.3 Activating transcription factor 6 (ATF6)
ATF6 is a sensor of ER stress involved in ER chaperone protein transcription.
ATH: ATF6 has been shown to mediate ox-LDL-induced cholesterol accumulation and apoptosis in cultured macrophages. C/EBP homologous protein (CHOP) is a key-signaling component of ER stress-induced apoptosis. The CHOP regulator ATF6 was up-regulated in ox-LDL treated cells. ATF6 silencing mitigated ox-LDL mediated CHOP upregulation, cholesterol accumulation and apoptosis in macrophages (Yao *et al.*, 2013).

5.4 Acyl-CoA synthetase 3 (ACLS3)
ACLS3 is an isoform of the long-chain fatty-acid-coenzyme A ligase family. It is a central element of the lipid synthesis and fatty acid metabolism.
ATH: Free fatty acids (FFAs) are known to promote atherosclerosis formation while eicosapentaenoic acid (EPA) in parts counteracts FFA effects on the vasculature. Palmitic acid (PA) increased and EPA diminished expression of TGF-beta family member bone morphogenetic protein 2 (BMP-2), the transcription repressor Msh Homeobox2 (Msx2), and the ant-apoptotic protein osteopontin in VSMC. ACSL3 expression was predominant in HASMC and ACSL3 silencing prevented PA mediated upregulation of BMP-2 and Msx2. It further attenuated PA induced calcium deposition and caspase activation (Kageyama *et al.*, 2013).

5.5 Perilipin 3 (PLIN3)
PLIN 3, an intracellular protein that physically spans around lipid droplets, is implicated in foam cell formation.

ATH: PLIN3 silencing reduced triacylglycerol (TAG), but not cholesterol content, in RAW 264.7 macrophages (Fan *et al.*, 2013).

5.6 Integrator Complex Subunit 6 (Int6)
Int6 is involved in the transcriptional regulation of small nuclear RNAs and is believed to have a role as a tumor suppressor gene.
ATH: Int6 is hypothesized to bind and target hypoxia inducible factor (HIF)-2α for degradation. HIF-2α encodes part of a transcription factor that is expressed in endothelial cells and is involved in the response to hypoxia leading to erythropoiesis via erythropoietin induction (Yamashita *et al.*, 2008). Int6 silencing increased angiogenesis in rat hindlimb muscles via up-regulation of HIF-2α induced angiogenic basic fibroblast growth factor and hepatocyte growth factor. This resulted in reduced gait disturbance (Okamoto *et al.*, 2011).

5.7 Formyl peptide receptor 2 (FPR2)
Various receptors including FPR2, TLR2, and TLR4 recognize serum amyloid A (SAA) that is involved in cholesterol transport and inflammation. FPR2 is a G-protein coupled receptor (GPCR) involved in foam cell formation (Lee *et al.*, 2013a; 2013b).
ATH: FPR2 was upregulated in peripheral blood mononuclear cells from patients with atherosclerosis. SAA stimulated macrophage foam cell formation via upregulation of scavenger receptor, LOX in an FPR2-dependent manner, which was inhibited by FPR2 silencing (Lee *et al.*, 2013a).

5.8 ATPase 7A (ATP7A)
ATP7A is an ATPase involved in copper level modulation and can be found in atherosclerotic blood vessels.
ATH: ATP7A silencing in THP-1 derived macrophages attenuated cell-mediated oxidation of LDL and decreased expression and cytosolic phospholipases A2 alpha cPLA(2)alpha. Conversely, cPLA(2)alpha overexpression increased LDL oxidation, which was blocked by ATP7A silencing (Qin *et al.*, 2010).

5.9 Sphingosine kinase-1 (SPHK1) SPHK1 is involved in the phosphorylation of sphingosine to sphingosine 1-phosphate (S1P), which mediates various cellular functions including TNF-alpha signaling and NF-kappa-B activation (Zhi *et al.*, 2006).
ATH: Factor-X (FXa) regulated SPHK1 transcription, S1P formation, and cell division and migration of VSMC. In addition, FXa rapidly induced the activation of the small GTPase Rho A. SPHK1 silencing attenuated the mitogenic and chemotactic response of human VSMC to FXa (Bohm *et al.*, 2013).

5.10 CD32, p47(phox), TNF-α converting enzyme (TACE)
CD32 is an immunoglobulin Fc receptor found on macrophages and neutrophils that is involved in phagocytosis (Sondermann *et al.*, 1999). P47(phox) is a 47 kDa cytosolic subunit of the neutrophil NADPH oxidase complex, which produces superoxide anions (Hsich *et al.*, 2000; Patterson *et al.*, 1999). TACE, also known as disintegrin and metalloproteinase domain-containing protein (ADAM) 17 endopeptidase is the major TNF-α convertase, that cleaves the soluble TNF-α form from the transmembrane TNF-α (Bazzoni & Beutler, 1996).
ATH: The soluble lectin-like ox-LDL receptor-1 (sLOX-1) is implicated in atherosclerosis. It was hypothesized that C-reactive protein may induce sLOX-1 release via TACE activation. Silencing of CD32, p47(phox), or TACE decreased of CRP-induced sLOX-1 expression in THP-1 derived macrophages (Zhao *et al.*, 2011).

5.11 Proprotein convertase subtilisin/kexin type 9 (PCSK9)
Gain-of function mutations of the PCSK9 gene are associated with hypercholesterolemia and increased risk of cardiovascular events.
ATH: PCSK9 promotes inflammation and endothelial dysfunction in LDL receptor (LDLR) independent manner (Urban *et al.*, 2013). PCSK9 silencing human umbilical vein endothelial cells (HUVEC) decreased ox-LDL-induced apoptosis and PCSK9 expression, but not LOX-1 expression, possibly through Bcl/Bax-caspase 9-caspase 3-pathway involvement (Wu *et al.*, 2012). Efficacious silencing of PCSK9 could already be demonstrated in vivo (Frank-Kamenetsky *et al.*, 2008; Gupta *et al.*, 2010; Makinen & Yla-Herttuala, 2013).

6. Reactive Oxygen Species (Ros)

6.1 Cyclophilin A (CyPA)
CyPA is a ROS-induced enzyme that enhances inflammatory activity of vascular cells and thus contributes to atherosclerotic plaque formation (Wei *et al.*, 2013).
ATH: CyPA silencing in ECs inhibited TNF-α-induced apoptosis possibly through decreased caspase-3 expression (Wei *et al.*, 2013).

6.2 Xanthine oxidoreductase (XOR)
XOR generates reactive oxygen species and is involved in macrophage foam cell formation and atherosclerosis development.
ATH: XOR silencing suppressed transformation of low-density lipoprotein (LDL) or very low density lipoprotein (VLDL) treated macrophages into foam cells (Kushiyama *et al.*, 2012). ABCA1 expression was upregulated in response to XOR silencing (Kushiyama *et al.*, 2012).

6.3 Meprin alpha
Meprins are proteinases that hydrolyze biologically active peptides, cytokines, ECM and cell-surface proteins and believed to have a role in vascular disease.
ATH: ox-LDL increased ROS formation and epidermal growth factor receptor (EGFR) in J774a.1 macrophages. Meprin alpha enhanced ox-LDL-induced plaque and ROS formation through EGFR transactivation. Meprin alpha silencing inhibited ROS production and EGFR activation in macrophages. (Gao *et al.*, 2013).

6.4 Antioxidant-1 (Atox1)
Atox1 is copper transport protein and is involved in copper-induced cell growth.
ATH: Atox1 was expressed in wire injured mouse arteries, where it co-localized with the copper transporter ATP7A. Atox1 silencing inhibited platelet derived growth factor (PDGF)-induced VSMC migration by inhibiting lamellipodia formation (Kohno *et al.*, 2013).

6.5 Vascular peroxidase 1 (VPO1) and Nox gp91(phox)
ATH: Myeloperoxidase (MPO) is involved in the development of atherosclerosis through the generation of radical oxygen species. VPO is a newly discovered member of the peroxidase family that is mainly expressed in vascular EC and SMC. Gp91(phox) is a catalytic subunit of NOX expressed in endothelial cells (Jones *et al.*, 1996).
ATH: ox-LDL induced EC apoptosis and VPO1 expression in EC, up-regulated protein expression of Nox gp91(phox) subunit and phosphorylation of p38 MAPK. All these effects of ox-LDL were inhibited by VPO1 and Nox gp91(phox) subunit gene silencing (Bai *et al.*, 2011).

7. Transcription Factors

7.1 Myocardin-related transcription factor (MRTF)-A
MRTF-A is a transcriptional regulator of SMC phenotype and deemed responsible oxLDL related endothelial injury.
ATH: MRTF-A silencing diminished ox-LDL induced ICAM-1 upregulation in EC (Fang *et al.*, 2011).

7.2 Lipopolysaccharide-induced tumor necrosis factor-alpha factor (LITAF) and Signal Transducer and Activator of Transcription (STAT)6B
LITAF increases TNF-alpha expression binding to the TNF-alpha promoter region and appears to be involved in p53-induced apoptosis. STAT6B is a transcription factor and member of the STAT family and is involved in IL-4 mediated anti-apoptotic signaling. LITAF and STAT6B promoted the inflammatory response by mediating LPS-induced CCL2 expression in macrophages (Tang *et al.*, 2011).
ATH: VEGF is essential in angiogenesis. VEGF and its isoforms elicit effects on endothelial and inflammatory cells and have been implicated in inflammatory diseases such as atherosclerosis. LITAF and/or mSTAT6B silencing in murine macrophages reduced VEGF expression and inhibited LPS-induced VEGF secretion. Angiogenesis was reduced in STAT6B-silenced wild-type animals compared

with control animals (Tang *et al.*, 2013).

7.3 cAMP response element-binding protein (CREB) and Ras-related C3 botulinum toxin substrate 1 (Rac1)
CREB is a transcription factor involved in cell proliferation, survival, and differentiation (Lee *et al.*, 2012). Rac1 is a member of the Rho family of small GTP-binding proteins that regulates migration through assembly of actin filaments (Small *et al.*, 1999).
ATH: VSMC migration from the media into intima contributes to the development of atherosclerosis. CREB silencing in VSMC inhibited TLR1/2 agonist Pam3CSK4-induced IL-6 production and migration. Further, Rac1 silencing also inhibited Pam3CSK4-induced VSMC migration. CREB activation led to IL-6 production, which enhanced VSMC migration via Rac1-mediated actin cytoskeletal reorganization (Lee *et al.*, 2012).

7.4 NonO and DJ-1
AA: TNF-alpha is known to degrade and decrease arterial collagen and has been linked to vulnerable plaque and aortic aneurysms (Zhang *et al.*, 2007). The transcription factor NonO protein binds to the human prolyl-4 hydroxylase alpha1 (P4H alpha1) promoter region. P4H alpha1 is a key enzyme involved in collagen metabolism. TNF-alpha suppressed P4Halpha1, which was reversed by NonO silencing (Zhang *et al.*, 2007). Furthermore, TNF-alpha oxidized DJ-1, a transcription factor protective against oxidative stress and cell death. DJ-1 silencing abrogated TNF-α-induced NonO-P4Halpha1 interaction and its suppression (Zhang *et al.*, 2008).

8. Proteinase/Peptidases

8.1 Chymase
Chymase is a member of the serine proteinase family that can be found in mast cells and basophile granulocytes. Chymase is known to convert angiotensin I to angiotensin II and thus is believed to contribute to hypertension and atherosclerosis.
ATH: Chymase is an activator of MMP9 and chymase activity was believed to affect plaque vulnerability in a hamster model of atherosclerosis. Lentiviral mediated silencing of the chymase remarkably enhanced atherosclerosis plaque stability (Guo *et al.*, 2013).

8.2 Tryptase
Tryptase is a member of the serine proteinase family and is found in mast cells. It is the most abundant mast cell granule protein and is considered a marker of mast cell activation. Tryptase has been linked to atherosclerosis plaque development.
ATH: Tryptase silencing decreased plaque expression of tissue plasminogen activator (tPA), CD31, CD34, VEGF, plaque size and protected from plaque hemorrhage in ApoE (-/-) mice (Zhi *et al.*, 2013).

8.3 Dipeptidyl peptidase 4 (DPP4) and DPP9
DPP are serine proteinases found in most cell types that have been linked to diabetes mellitus and cancer development.
ATH: Aside from being a therapeutic target in type 2 diabetes, DPP4 may play a role in atherosclerosis. While DPP4 was only observed in EC of plaque neovessels in half of the specimens, DPP8 and DPP9 were widely expressed in macrophage-rich plaques regions. In parallel, DPP9 silencing reduced TNF-α and IL-6 secretion in macrophages (Matheeussen *et al.*, 2013).

8.4 Proto-oncogene tyrosine-protein kinase Src (c-Src)
c-Src, a tyrosine kinase originally described in cancer, has known roles in cell migration, proliferation, survival as well as angiogenesis.
ATH: Endothelial dysfunction is one of the earliest pathophysiologic changes leading to atherosclerosis. ADAM15 is a metalloproteinase that regulates endothelial permeability.

ADAM15-induced barrier dysfunction is associated with dissociation of endothelial adherens junctions (VE-cadherin/γ-catenin). cSrc silencing revealed cSrc to be a mediator of the endothelial barrier effects of ADAM15 (Sun *et al.*, 2012).

9. Protein Kinases and Associated Proteins

9.1 Sprouty homolog 4 (Spry 4)
ATH: Many cytokines signal through receptor tyrosine kinases (RTK). PI3K/Akt and MAPK/ERK pathways regulate phenotypic transition of VSMC and various other cell functions. Spry proteins regulate RTK signaling feedback. Spry4 is an inhibitor of Akt and MAPK signaling and has been linked to testicular and germ cell cancers. Further, Spry4 silencing resulted in upregulation of myocardin in VSMC, a master gene for contractile VSMC phenotype maintenance (Yang *et al.*, 2013a).

10. Cell Surface Molecules/Membrane Receptors/Membrane Associated Proteins

10.1 Annexin A2 (A2t)
A2t is a phospholipid-binding protein involved in cell motility and other cell-matrix interactions.
ATH: Proinflammatory stimuli lead to activation of EC, which results in expression of cellular adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) on the cell surface. Plasmin induced ICAM-1 expression in HUVEC through A2t activation, followed by Akt/NF-κB, p38 MAPK and ERK1/2 signaling. A2t silencing blocked plasmin-induced ICAM-1 upregulation in HUVEC (Li *et al.*, 2013).

10.2 Reticulocalbin 2, EF-hand calcium binding domain (Rcn2)
The genetic locus Ath29 confers atherosclerosis susceptibility. Rcn2 is associated with Ath29 and has a role in endoplasmatic reticulum Ca(2+) signaling.
ATH: Rcn2 silencing led to decreased oxidized phospholipid-induced VCAM-1 and MCP-1 expression in EC (Manichaikul *et al.*, 2011).

10.3 Alpha receptors (alpha1B-ARs and alpha1D-AR)
Norepinephrine (NE) is a potent activator of alpha1B-ARs and alpha1D-AR.
ATH: In a mouse model of vena cava segment to carotid arteries grafting, mechanical shear stress (SS) increased alpha1B-AR and alpha1D-AR expression. Combined SS and NE stimulation lead to peak activation of ERK and Ki67 expression in VSMC. Alpha1B-ARs and alpha1D-ARs silencing partially inhibited these effects (Liu *et al.*, 2013b).

11. Miscellaneous

Cytokine induced apoptosis inhibitor (CIAPIN1)
CIAPIN1 is a cytokine and growth factor induced anti-apoptosis regulator associated with cancer angiogenesis (Yan *et al.*, 2009).
ATH: CIAPIN1 silencing in VSMC decreased proliferation, migration and promoted apoptosis of VSMCs via modulation of Bcl-2 and Bax (Yang *et al.*, 2013b).

BRCA1 and BRCA-1 associated protein gene (BRAP)
Specific BRCA1 mutations are known to cause hereditary breast and ovarian cancers. BRCA is involved in DNA repair and cellular stress response.
ATH: BRCA1-overexpression in ApoE null mice led to less aortic plaque burden, diminished ROS generation and ameliorated macrophage infiltration compared to control animals (Singh *et al.*, 2013). BRCA1 gain of function also enhanced angiogenesis in a murine model of hindlimb ischemia (Singh *et al.*, 2013). Certain BRAP single nucleotide polymorphisms are associated with an increased myocardial infarction risk in the Japanese population (Ozaki *et al.*, 2009). BRAP was further detected in SMC and macrophages of atherosclerotic plaques (Ozaki *et al.*, 2009). BRAP silencing in EC blocked NF-kappaB activation (Ozaki *et al.*, 2009). BRAP silencing also decreased LPS induced HASMC proliferation and MCP-1 and IL-8 secretion (Liao *et al.*, 2011).

K(Ca)3.1 channel
K(Ca)3.1 channel is a voltage independent potassium channel activated by intracellular calcium. It was found upregulated in VSMC, macrophages, and T-lymphocytes of atherosclerotic plaques in ApoE null mice (Toyama *et al.*, 2008).
ATH: Advanced glycemic endproducts (AGEs)-induced VSMC migration and proliferation coincided with K(Ca)3.1 channel upregulation and could be reversed by K(Ca)3.1 channel silencing. K(Ca)3.1 channel expression was modulated by ERK1/2, P38-MAPK and PI3K (Zhao *et al.*, 2013).

WNT1-inducible-signaling pathway protein 1 (WISP-1 or CCN4)
WISP-1 or CCN4 is a member of the CCN protein family, which controls cell development and survival in multiple systems and is an ECM-associated secreted protein.
ATH: TNF-alpha increased CCN4 expression in VSMC. CCN4 upregulated VCAM1 and secretory/synthetic SMC phenotype markers osteopontin and elastin. CCN4 silencing inhibited VSMC proliferation, and reversed CCN4 mediated osteopontin and elastin upregulation (Liu *et al.*, 2013a).

Connective Tissue Growth Factor (CTGF)
CTGF has profibrotic properties and is involved in fibroblast proliferation, angiogenesis and ECM synthesis. Some downstream effects of TGF-β are mediated by CTGF.
ATH: VSMC exposed to high-glucose (HG) media showed increased levels of CTGF, enhanced proliferation and increased ECM accumulation. CTGF silencing reduced cell proliferation and ECM component accumulation. Further, ERK1/2 silencing inhibited HG-induced CTGF mediated effects (Ha *et al.*, 2013).

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