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Current siRNA Targets in Atherosclerosis and Aortic Aneurysm

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Abstract

Atherosclerosis (ATH) and aortic aneurysms (AA) remain challenging chronic diseases that confer high morbidity and mortality despite advances in medical, interventional, and surgical care. RNA interference represents a promising technology that may be utilized to silence genes contributing to ATH and AA. Despite positive results in preclinical and some clinical feasibility studies, challenges such as target/sequence validation, tissue specificity, transfection efficiency, and mitigation of unwanted off-target effects remain to be addressed. In this review the most current targets and some novel approaches in siRNA delivery are being discussed. Due to the plethora of investigated targets, only studies published between 2010 and 2014 were included.

Introduction

Cardiovascular diseases such as atherosclerosis (ATH) and aortic aneurysms (AA) remain associated with high morbidity and mortality. Novel molecular therapies need to be investigated to more effectively treat or prevent these conditions. Small interfering RNA (siRNA) is a member of the non-coding RNA family, which includes ribosomal RNA, transfer RNA, microRNA, and several others. Since its discovery, siRNA rapidly permeated the world of science, finding its place in laboratories as a tool for diagnostic or therapeutic nonviral gene silencing (Dorsett and Tuschl, 2004; Elbashir *et al.*, 2001; Zamore *et al.*, 2000). First human clinical trials showed safety and efficacy of therapeutic siRNA delivery for the treatment of amyloidosis and liver cancer (Coelho *et al.*, 2013; Tabernero *et al.*, 2013).

However, siRNA target choice, off-target effects, and tissue/cell specific delivery still represent significant challenges that need to be addressed in order to optimize study outcomes.

Previously in the Prevent III trial, oligodeoxyribonucleotides that are chemically different from siRNA were used to repress cycle regulator elongation factor 2 (E2F) mRNA in human vein grafts. This large trial failed despite promising preclinical data and emphasized how

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crucial target choice is for the success of non-viral, nucleotide based gene therapy (Conte *et al.*, 2006). Given cellular signaling redundancy it might be necessary to silence multiple genes simultaneously and/or sequentially to achieve clinically detectable effects.

In this review, ATH and AA specific studies investigating siRNA targets were summarized (see also Tables 1 and 2). While in the majority of the listed studies in which siRNA was used as a possible therapeutic tool, some studies were included, in which siRNA was used for diagnostic purposes. Given the plethora of siRNA literature pertaining to vascular disease, especially ATH, the role of microRNA (miRNA) will be discussed at a different time, as it would exceed the scope of this review.

Methods

Combined PubMed searches that included the words atherosclerosis and siRNA or aortic aneurysm and siRNA were performed. Given the expansive literature on atherosclerosis and siRNA, and partial redundancy, only articles published between 2010 and 2014 were considered for this review. Since pathophysiology of AA shows some key similarities to ATH, we have also included target genes relevant in AA.

Atherosclerosis (ATH)

Atherosclerosis is the most common of the cardiovascular diseases contributing to major morbidity and mortality in the developed world. Several contributing factors including inflammation, endothelial dysfunction, dyslipidemia, diabetes, and hypertension contribute to the development of atherosclerosis. The ultimate manifestation of atherosclerosis that results in plaque development and blockade of blood vessels by recruitment of macrophages, foam cell formation, production of reactive oxygen species, smooth muscle proliferation, and extracellular matrix modulation has been extensively studied including therapies to prevent these events and processes. The number of siRNA-based studies that are aimed to curtail the development of atherosclerosis by targeting the contributing factors and co-morbidities has gone up exponentially and the literature is expanding with an ever-increasing library of potential targets. In the present review, we have included the most recently tested siRNA targets for the treatment and/or prevention of atherosclerosis and cataloged them according to the main pathophysiologic effect or cellular function that they are involved in. Naturally there will be overlap between the individual categories.

Aortic aneurysm (AA)

Aortic aneurysmal disease shares significant pathologic similarities with atherosclerosis. However, enzymatic degradation of the elastic laminae and extracellular matrix (ECM) of the arterial wall is an additional pathognomonic feature that leads to the characteristic weakening and dilation of the aorta. Atherosclerosis, uncontrolled hypertension, trauma, infection, and certain genetic diseases affecting elastin and collagen metabolism are considered etiologies of AA. Conservative management therefore includes medical management of hypertension and hypercholesterolemia, but also the antibiotic doxycycline is being evaluated for its efficacy in treating AA (Petrinec *et al.*, 1996; Thompson *et al.*, 1999; 1998).

In order to better understand the morphologic changes of the affected arteries, extensive molecular analyses have been conducted. Of particular interest has been the role of matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitors of matrix metalloproteinases (TIMP); MMPs are known to degrade ECM and a dysbalance of MMPs and TIMPs with a shift towards greater MMP activity is believed to contribute to the pathophysiology of arterial aneurysms (Davis *et al.*, 1998; Newman *et al.*, 1994a; 1994b; Palombo *et al.*, 1999; Thompson *et al.*, 1999; Thompson and Baxter, 1999). In this review siRNA experiments targeting MMPs and other potential targets deemed relevant for AA formation were included.

Immune Mediators

Immune mediators that lead to the inflammatory milieu contributing to the pathophysiology of ATH and AA are widely investigated. Following are key targets that were silenced with siRNAs in order to yield a more favorable inflammatory signaling pattern and mitigate ATH and AA.

Toll-like receptor (TLR)

Toll-like receptors are cell membrane pattern recognition receptors involved in the innate immune system and found on macrophages, dendritic, and other cells.

The activation of TLR4 by lipopolysaccharide (LPS) can create an inflammatory milieu that promotes the development of atherosclerosis and diabetes. TLR4 is involved in macrophage foam cell formation, which is a key step in atherosclerotic plaque pathology. TLR4 silencing in vascular smooth muscle cells (VSMC) revealed that effects of anti-inflammatory drugs such as fenofibrate, a Peroxisome proliferator-activated receptor-alpha (PPAR)-α agonist and rosiglitazone, a PPAR-gamma (PPAR-γ) agonist on LPS-mediated inflammation in VSMCs were dependent on TLR4 (Ji *et al.*, 2011; 2010). Plasmid mediated TLR4 silencing in U937 pre-monocytes decreased nuclear factor-kappaB (NF-κB) activity, the secretions of monocyte chemoattractant protein-1 (MCP-1) and the chemokine interleukin-8 (IL-8) in response to oxidized low density lipoprotein (ox-LDL). NF-κB, MCP-1, and IL-8 are all known inflammatory mediators contributing to a myriad of diseases (Geng *et al.*, 2010). Bone morphogenetic protein-2 (BMP-2) plays an important role in atherosclerotic vascular calcification. TLR2 and TLR4 silencing reduced oxidized Low Density Lipoprotein (oxLDL)-induced BMP-2 expression in human coronary artery endothelial cells (HCAEC) (Su *et al.*, 2011). C-reactive protein (CRP) may induce inflammatory responses leading to ATH via a TLR4-dependent signaling pathway, Angiotensin II Type I receptor-p38 mitogen-activated protein kinase-TLR4-Protein Kinase C-alpha (AT-1R-p38 MAPK-TLR4-PKCalpha) in VSMC. TLR4 silencing reversed these pro-inflammatory effects of CRP (Liu *et al.*, 2010). Dysregulated expression of matrix metalloproteinases (MMPs) are known to play a role in various cardiovascular diseases including ATH and AA. TLR2 and TLR4 silencing reduced MMP-2 and MMP-9 gene expression in mouse aortic EC (Cheng *et al.*, 2011).

Mast cells can infiltrate into the atheromatous plaque and secrete various pro-inflammatory cytokines. TLR4 silencing in cultured human mast cells mitigated ox-LDL mediated

upregulation of TLR4 and MCP-1, as well as Tumor Necrosis Factor-alpha (TNF-α) and IL-6 secretion. Moreover, TLR4 silencing attenuated ox-LDL stimulated nuclear translocation of NF-κB, and MAPK phosphorylation (Meng *et al.*, 2013).

TLR4 mRNAs often contain AU-rich elements (AREs) in their 3′-untranslated regions (3′UTR), which have a high affinity for RNA-binding proteins such as human antigen R (HuR) that regulate TLR4 expression in human aortic smooth muscle cells (HASMCs). Knockdown of HuR inhibited LPS-induced TLR4 mRNA stability in HASMCs (Lin *et al.*, 2006).

Mammalian Target of Rapamycin (mTOR)

The mammalian target of rapamycin (mTOR) is an intracellular regulator of multiple cellular functions including mitosis, migration, and differentiation (Wang *et al.*, 2013b).

ATH: mTOR has been believed to be involved in the development of atherosclerotic plaques. mTOR silencing in mice decreased macrophage presence and inhibited the progression of atherosclerotic plaques along with decreasing expression of plaque instability. Silencing of mTOR further decreased expression of MMP2, MCP-1, and tissue factor (TF) (Wang *et al.*, 2013b). Inflammation disrupts low-density lipoprotein receptor (LDLr) pathway feedback regulation through the activation of the mTOR pathway in atherosclerosis. Inhibition of the mTOR pathway by mTOR siRNA prevented foam cell formation *in vitro* and decreased levels of LDLr, in lipopolysaccharide stimulated VSMC (Ma *et al.*, 2013a). Silencing of mTOR and its associated proteins rictor and raptor, inhibited the upregulation of TLR4 expression. Further, inhibition of ox-LDL induced mTOR activation reduced TLR4 expression, and improved the impaired lipid efflux (Yu *et al.*, 2011).

Chemokine receptors

Increased chemokine receptor expression is known to occur in almost all cardiovascular diseases. Immune cell trafficking, a major process involved in ATH and AA, is regulated by the expression of chemokine receptors not only on immune cells but also on vascular cells. Following chemokine receptors were targeted for siRNA therapy or diagnostics.

C-C chemokine receptor type 2 (CCR2)—CCR2 is the receptor for MCP-1 that itself is involved monocyte chemotaxis.

ATH: Inflammatory monocytes but not the non-inflammatory subset depend on the chemokine receptor CCR2 for localization to injured tissue. CCR2 siRNA carrying nanoparticles administered systemically in mice, localized to monocytes, and were rapidly cleared from the blood. CCR2 silencing in monocytes prevented their accumulation in sites of inflammation in mice after myocardial infarction (Leuschner *et al.*, 2011; Majmudar *et al.*, 2013).

AA: CCR2 is also involved in AA formation. siRNA-induced inhibition of CCR2 in leukocytes inhibited aneurysm formation in mice with disruption of the MCP-1/CCR2 signaling pathway (de Waard *et al.*, 2010).

Chemokine (C-X3-C motif) receptor 1 (CX3CR1)—ATH: The chemokine receptor CX3CR1 is expressed in blood monocytes, dendritic cells (DC), and SMC and is associated with atherosclerotic plaque pathology (Landsman *et al.*, 2009; Liu *et al.*, 2011). CX3CR1 and its ligand fractalkine (CX3CL1) are believed to mediate anchorage and chemotaxis between macrophages and SMCs. The interaction of LPS-activated monocytes with SMC increased the expression of TNF-α, MMP-9, and CX3CR1 in both cell types. CX3CR1 knockdown before cell-cell interaction significantly decreased TNF-α, MMP-9, and CX3CR1 upregulation (Butoi *et al.*, 2011). Separately, CX3CR1 silencing has shown an inhibition of interactions between DC and EC (Liu *et al.*, 2011).

C-X-C chemokine receptor type 7 (CXCR7)—While CXCR7 is not expressed in normal blood leukocytes, its role in leukocytes in disease states is not fully understood (Ma *et al.*, 2013b). CXCR7 is considered a new receptor for stromal cell-derived factor-1 (SDF-1). SDF-1 is known to induce leukocyte chemotaxis including macrophages.

ATH: CXCR7 was expressed in macrophage positive areas of aortic atheromas of apolipoprotein E (ApoE)-null mice. Expression was more in M1 macrophages than in the M2 phenotype. CXCR7 was associated with an SDF-1 mediated pro-inflammatory signaling. CXCR7 silencing suppressed macrophage phagocytic activity (Ma *et al.*, 2013b).

Endoplasmic reticulum (ER) stress transducers protein kinase RNA-like endoplasmic reticulum kinase (PERK) and inositol-requiring protein 1 (IRE-1)

PERK inhibits translation in response to ER stress and the protein kinase IRE1 is involved in the response to ER based stress signals.

ATH: The high mobility group 1B protein (HMGB1) mediated chronic inflammatory responses in EC is critical for development of atherosclerosis. HMGB1 induced a dose-dependent activation of ER stress transducers (PERK and IRE-1) in EC. PERK or IRE1 silencing suppressed HMGB1-mediated intercellular cell adhesion molecule-1 (ICAM-1) and P-selectin (Luo *et al.*, 2013). PERK-silencing in RAW264.7 macrophages inhibited ox-LDL-induced apoptosis (Yao *et al.*, 2013).

MCP-1 secretion was increased by TNF-like weak inducer of apoptosis (TWEAK) increased in a cell line derived from human acute monocytic leukemia cells. This could be blocked by HMGB1 silencing (Moreno *et al.*, 2013).

SI00 alarmins S100A9 and S100A4

S100A4, a member of the S100 calcium-binding protein family with a role in cancer cell metastasis, is highly expressed in synthetic SMC, while it is barely detectable in contractile SMC (Boye and Maelandsmo, 2010; Brisset *et al.*, 2007). S100A9 is secreted by neutrophils and monocytes/macrophages and has been linked to cardiovascular disease (Cotoi *et al.*, 2014). S100A9 stimulates neutrophil activity and promotes phagocytosis (Simard *et al.*, 2011).

ATH: The periodontal pathogen, *Porphyromonas gingivalis*, is involved in atherosclerosis and aortic intimal hyperplasia. S100A9 was upregulated in aortic SMC in response to *P.*

gingivalis. Exposure of aortic SMC to the supernatant of plasma incubated with *P. gingivalis* induced a proliferative phenotype, which could be attenuated by S100A9 silencing (Inaba *et al.*, 2009). Plaque calcification is linked to macrophage accumulation. Silencing of S100A9 *in vitro* reduced macrophage matrix vesicle calcification (New *et al.*, 2013).

AA: S100A4 is found in the aortic wall in human thoracic aortic aneurysms. S100A4 silencing decreased VSMC proliferation and MMP-2 and MMP-9 expression (Cao *et al.*, 2013).

Lipid Metabolism Signaling

Liver X receptor alpha (LXRalpha)

LXRalpha belongs to a nuclear receptor superfamily that controls macrophage function, including lipid homeostasis and inflammation (Chawla *et al.*, 2001).

ATH: TLR2 ligand Pam(3)CSK(4) mediated TLR2 activation led to upregulation of ATP binding cassette transporter (ABCA1), a membrane bound regulator of cholesterol efflux in macrophages (Park *et al.*, 2013a). For a while there has been a controversy whether ABCA1 promotes or protects against atherosclerosis. Recent studies suggest that lower ABCA1 levels are associated with increased plaque burden (Bochem *et al.*, 2013; Liu *et al.*, 2012a).

Oxidized low-density lipoprotein receptor 1 (LOX-1)

LOX-1 internalizes and degrades ox-LDL and may have a role as scavenger receptor involved in Fas-induced apoptosis.

ATH: Stretch stress and ox-LDL could each induce activation of extracellular related kinase (ERK) 1/2 and Ki-67 in VSMCs. Knockdown of LOX-1 inhibited these effects (Zhang *et al.*, 2013b). In EC Angiotensin II (AngII) treatment induced inflammation, as indicated by upregulation of VCAM-1, MCP-1, and activation of NF-κB and LOX-1. LOX-1 silencing decreased AngII-induced VCAM-1 production (Wang *et al.*, 2013a).

Apolipoprotein B (apoB)

apoB is the main apolipoprotein of chylomicrons and LDL and is involved in lipoprotein shuttling throughout the body to the tissues.

ATH: Increased serum apoB and associated LDL levels are associated with an increased risk of coronary disease. apoB silencing in mice showed lipid-lowering effects for over three weeks (Tadin-Strapps *et al.*, 2011). Liver specific apoB100 silencing could be achieved by conjugation of siRNA to tocopherol (Makinen and Yla-Herttuala, 2013; Nishina *et al.*, 2008).

Reactive Oxygen Species (Ros)

NADPH oxidase 1 (Nox1), NADPH oxidase, EF-hand calcium binding domain 5 (Nox5), and Nox4

NADPH oxidases (Nox1, Nox3, Nox4, Nox5) are membrane bound enzymes involved in the generation of radical oxygen species and therefore implicated in cardiovascular disease.

ATH: Hyperglycemia induced Nox1 in EC. Nox1 silencing in EC decreased hyperglycemia induced MCP-1, VCAM-1, connective tissue growth factor (CTGF), collagen IV, and fibronectin expression (Gray *et al.*, 2013). Nox1 silencing in VSMC prevented thromboxane A(2) mimetic U46619 mediated increase of IL-1beta-induced monocyte adhesion (Bayat *et al.*, 2012). TNF-α and Nox-5 silencing inhibited AngII induced human umbilical arterial and venous EC chemokine CX(3)CL1 expression (Rius *et al.*, 2013).

AA: Hyperhomocysteinemia (HHcy) has been linked to the formation of abdominal aortic aneurysm (AAA). HHcy increased AngII-mediated AAA formation in apoE-deficient mice. HHcy promoted adventitial fibroblasts transformation into myofibroblasts, increased aortic adventitial inflammation and IL-6 and MCP-1 secretion. Adventitial fibroblast activation was ameliorated by Nox4 silencing (Liu *et al.*, 2012b).

Transcription Factors

Nuclear factor-kappaB (NF-κB)

NF-κB is a DNA transcription regulating protein complex and powerful modulator of inflammation that plays a role in injury response and vascular diseases.

ATH: Interleukin-17 induced NF-κB dependent VCAM-1 expression in VSMC, which could be abrogated by NF-κB silencing (Zhang *et al.*, 2013a).

Kruppel like factor 4 (KLF4)

The transcription factor KLF4 is expressed in EC and in inflamed SMC that mediates inflammatory responses after vascular injury. KLF4 is also a regulator of macrophage activation and believed to be involved in SMC phenotype switching (Yan *et al.*, 2008).

ATH: Phenotypic switching of SMC from contractile to secretory plays a central role in atherosclerosis and restenosis. Human myocardin (Myocd) is the master gene regulator of SMC differentiation. The upstream repressor region PrmM is localized within the Myocd promoter. Klf4 significantly decreased Myocd mRNA, while Klf4 silencing abolished PDGF-BB-mediated repression of PrmM-directed gene expression in SMC (Turner *et al.*, 2013).

AA: Aortic KLF4 expression progressively increased in response to elastase perfusion in C57BL/6 mice. Loss of a KLF4 allele conferred protection from aneurysm formation. KLF4 silencing attenuated downregulation of SM marker gene expression *in vitro* (Salmon *et al.*, 2013).

ATH: Silencing of the transcriptional regulator EGR-1 ameliorated hyperglycemia induced expression of CD11c, chemokine (C-C motif) ligand 2, and inter-leukin-1beta in U937 macrophages (Kong *et al.*, 2013).

Matrix Metalloproteinases (MMPs)

Activation of matrix metalloproteinases (MMPs) and their isoforms leads to ECM remodeling, which facilitates VSMC invasion, a key event in cardiovascular disease development. Protein kinase B (Akt) is a potent upstream regulator of MMP expression, which will be discussed later in the review.

ATH: MMP-2 and MMP-9 silencing suppressed VSMC migration in an *in vitro* study of atherosclerosis (Johnson *et al.*, 2011; Kim *et al.*, 2012). MMP-9 silencing in EC provided cell protective effects through decreasing high glucose-induced damage to the mitochondria and chaperone proteins (Kowluru *et al.*, 2011).

AA: MMP-2 activity is regulated by the intrinsic tissue inhibitor of MMP-2 (TIMP-2). MMP-2 silencing decreased SMC invasion, but not proliferation, adhesion, or migration (Kimura *et al.*, 2010). Silencing of MMP-9 and −12 in VSMC decreased beta-catenin signaling and proliferation possibly via a cyclin D1 involving mechanism (Dwivedi *et al.*, 2009).

Protein Kinases

Extracellular signal-regulated kinases 1/2 (ERK1/2)

Extracellular signal-regulated kinases are members of the mitogen-activated protein kinase (MAPK) family that mediate proliferation and differentiation signals in different cell types.

ATH: The proinflammatory cytokine IFN-gamma is involved in atherosclerosis and its cellular signaling is predominantly transmitted through STAT1. ICAM-1, MCP-1, IP-10, and MIP-1β upregulation by IFN-γ is ERK1/2-dependent. Further, ERK1/2 silencing in macrophages attenuated IFN-γ-induced ox-LDL uptake (Li *et al.*, 2010). Further, ERK1/2 silencing reversed high-glucose induced CTGF mediated proliferation and ECM production in VSMC (Ha *et al.*, 2013).

AA: ERK signaling is relevant for MMP activation during AAA formation. ERK1/2 silencing in elastase treated murine VSMC reduced MMP2 formation (Ghosh *et al.*, 2012).

c-Jun

c-Jun is a component of the AP-1 signaling pathway and involved cell cycle regulation and cell differentiation.

ATH: Interleukin-6 (IL-6) is a cytokine pivotal for ECM metabolism. IL-6 decreased P4Halpha1, which is required for collagen synthesis, and collagen and SMC contents in atherosclerotic mouse arteries. IL-6 mediated P4Halpha1 downregulation in hASMC occurred via the RAF-MEK1/2-ERK1/2 MAPK pathway. c-Jun silencing in hASMC mitigated IL-6-induced P4Halpha1 downregulation (Zhang *et al.*, 2012). Receptor activator

of NF-κB ligand (RANKL) plays a role in the thrombogenicity of atherosclerotic plaques. c-Jun and early growth response protein 1 (Egr-1) silencing in macrophages attenuated RANKL-induced tissue factor (TF) expression. In macrophages RANKL induced TF through AP-1 and Egr-1 via JNK and ERK1/2 pathways (Kim *et al.*, 2010).

c-Jun NH2-terminal kinases (JNK, JNK2)

JNK is a member of the MAPK family involved in cellular stress signaling and gene expression in EC in response to flow alterations (Wang *et al.*, 2011). Ischemia-reperfusion injury induced cell apoptosis is in part mediated by JNK (Xu *et al.*, 2010).

ATH: Serum amyloid A (SAA) upregulated pentraxin 3 (PTX3), a part of the innate immunity via formyl peptide receptor-like 1 (FPRL1), which overall enhanced proinflammatory processes. SAA induced PTX3 production was attenuated by JNK silencing (Dong *et al.*, 2011). The ability of EC to align in the direction of flow has shown to correlate with protection from atherosclerosis in these regions. JNK2 silencing in EC inhibited alignment in response to shear stress (Hahn *et al.*, 2011). Interestingly, JNK silencing in EC under low shear stress attenuated NF-κB activity and VCAM-1 expression (Wang *et al.*, 2011).

AngII has shown to raise leptin levels in adipocytes and ROS in VSMC, which in turn promotes atherosclerosis. JNK silencing attenuated AngII-induced leptin and phospho-JNK protein expression and diminished AngII-induced VSMC migration and proliferation (Shyu *et al.*, 2012).

AA: In a mouse model of abdominal AA (AAA) males developed larger AAA compared with females with higher levels of JNK1, proMMP2, and proMMP9. JNK1 or JNK2 silencing in VSMC decreased MMP activity, while combined JNK 1 and 2 silencing diminished all MMP activity *in vitro* (DiMusto *et al.*, 2012).

p38 mitogen-activated protein kinase family (p38 MAPK)

p38 MAPK are members of the mitogen-activated protein kinase family that respond to stress stimuli and play a role in cell differentiation and apoptosis.

ATH: p38 MAPK is implicated in the development of atherosclerosis. p38 MAPK silencing reduced the LDL-induced cholesterol accumulation in macrophages. LDL cholesterol loading-induced inhibition of autophagy was prevented by p38 MAPK silencing (Mei *et al.*, 2012).

PPARgamma ligands have shown to ameliorate AngII-induced atherosclerotic changes. AngII suppressed PPARgamma expression and activity in VSMC in a TGF-beta1 dependent fashion. TGF-beta1 secretion in response to AngII required ROS mediated EGFR kinase activation. p38 MAPK silencing inhibited both AngII- and TGF-beta1-induced PPARgamma reduction (Subramanian *et al.*, 2012).

Protein kinase B (AKT)

AA: Akt is a serine/threonine protein kinase with several isoforms, which are involved in abnormal vascular remodeling through modulation of apoptosis, proliferation, migration, glucose metabolism, and other functions (Jung *et al.*, 2000).

Akt siRNA decreased proMMP2 and proMMP-9, as well as active MMP-2 secretion from elastase-treated VSMC (Ghosh *et al.*, 2014). Interestingly, male human abdominal AA specimens showed higher ratios of phosphorylated AKT/AKT ratios than those from women, implying that differential levels of Akt phosphorylation may be important in sex differences in abdominal AA (Ghosh *et al.*, 2014).

In contrast, deficiency of isoform Akt2 in mice rendered animals more susceptible to AA formation possibly due to an observed increase in MMP-9 and decrease in TIMP-1 activity. In addition Akt2 and phospho-Akt levels were decreased in human thoracic AA specimen (Shen *et al.*, 2013). These opposing observations illustrate the complexity of Akt biology and may also be partially explained by the differential embryonic composition of the aortic segments investigated.

Protein kinase C (PKC) alpha/beta/delta (PKCα/β/δ)

PKC is a family of protein kinases involved in a variety of cellular functions such as apoptosis and endothelial cell proliferation.

PKCα activation can elicit proangiogenic effects, but also increase endothelial permeability (Harrington *et al.*, 1997; Vandenbroucke St Amant *et al.*, 2012).

PKCβ upregulation has been shown to cause vascular dysfunction, by negatively affecting endothelial barrier function, and decreased Akt mediated eNOS expression in response to insulin (Kong *et al.*, 2013; Naruse *et al.*, 2006; Vuong *et al.*, 1998). PKCβ can accelerate diabetic atherosclerosis in mice by modulating macrophage activity and CD11c expression (Kong *et al.*, 2013).

PKCδ increased VSMC adhesion and migration in response to injury and upregulated expression of cholesterol receptors CD36 and SR-A (Kamiya *et al.*, 2007; Lin *et al.*, 2012).

ATH: CRP treated macrophages showed increased levels of PKCα and PKCδ with subsequent upregulation of IL-1, MCP-1, TNF-alpha, and IL-6. PKCα and PKCδ silencing reversed these effects (Jialal and Devaraj, 2012; Jialal *et al.*, 2013a; 2013b). PKC regulated apoE secretion independent of ABCA1. PKCα/β silencing in macrophages inhibited apoE secretion (Karunakaran *et al.*, 2013). PKCδ elicited pro-apoptotic signals in various cells and has a role in the stress response to ox-LDL. PKCδ silencing in VSMC protected against ox-LDL-induced apoptosis (Larroque-Cardoso *et al.*, 2013).

Janus kinase/signal transducer and activator of transcription (JAK/STAT)

The JAK/STAT system binds to transmembrane receptors and aids in transmitting extracellular signals into the nucleus. Leukocytes show activation of several JAK/STAT pathways, which thus are believed to involve immune functions.

ATH: Advanced oxidation protein products (AOPP) are suspected to promote coronary artery disease. In T-helper-1 (THP-1)-derived foam-like cells, AOPP decreased LXRalpha and ABCA1 via JAK/STAT, which in turn inhibited cholesterol efflux.

JAK/STAT silencing could block these effects of AOPP in THP-1 cells (Mo *et al.*, 2011). Tissue factor (TF) has a central role in the coagulation cascade and is also involved in neointima and atherosclerosis development. JAK2 silencing by siRNA inhibited TLR2 ligand Pam3CSK4 induced TF expression in macrophages. Pam3CSK4 stimulated STAT3 phosphorylation (S727), while STAT3 siRNA reduced Pam3CSK4-induced TF expression (Park *et al.*, 2013b).

Cell Surface Molecules/Membrane Receptors/ Membrane Associated Proteins

Platelet endothelial cell adhesion molecule 1 (PECAM-1) or CD31 and fibronectin (FN)

PECAM-1 or CD31 is a surface antigen found on many cell types such as EC, neutrophils, monocytes, and platelets and is involved in leukocyte migration, angio-genesis, and other cell functions.

FN is a glycoprotein and crucial component linking ECM to cell membrane bound integrins. FN plays central roles in cell differentiation, migration, proliferation, and other functions.

ATH: As discussed earlier, JNK silencing attenuated NF-κB activity and VCAM-1 expression in HUVECs under low shear stress. Furthermore, CD31 siRNA reduced p-JNK and VCAM-1 levels in the setting of low shear stress. In the setting of low shear stress JNK may promote atherosclerosis through a CD31-dependent pathway and by modulating NF-κB and VCAM-1 (Wang *et al.*, 2011).

It was demonstrated that CD31 mediated FN deposition in response to atheroprone flow, which in turn increased NF-κB activity and VCAM1 expression in EC. CD31 silencing in EC blocked FN deposition, decreased NF-κB activation, and lowered VCAM1 expression. Additionally, FN silencing also reduced NF-κB activity, which was reversed by exogenous FN (Feaver *et al.*, 2010).

Ephrin receptors ephrin A2 (EphA2) and ephrin A4 (EphA4)

Eph receptors are cell membrane bound and are activated in response to injury. EphA receptor and EphA ligand expression is induced by various proinflammatory mediators (Funk *et al.*, 2012).

ATH: EphA2 and its ligand EphA1 are highly expressed in EC of atherosclerotic plaques of mice and humans. EphA2 silencing diminished VCAM-1 expression in response to EphA1 and ox-LDL (Funk *et al.*, 2012). In a separate study EphA4 and EphA1 were found in most cells within human atherosclerotic plaques. EphA4 silencing blocked EphA1-induced monocyte adhesion to EC as well as stress fiber formation (Jellinghaus *et al.*, 2013).

Caveolin (Cav-1)

Cav-1 is a component of the plasma membrane caveolae and is involved in macrophage inflammation, adhesion, and phagocytosis and thus is believed to influence the development of IH and atherosclerosis (Fu *et al.*, 2012; Luo *et al.*, 2010).

ATH: Cav-1 silencing reduced cholesterol efflux in macrophages (Hu *et al.*, 2010). Cav-1 promoted monocyte to macrophage differentiation via EGR-1. Cav-1 gain of function in macrophages increased ICAM-1 and CD11b, while Cav-1 silencing reduced these (Fu *et al.*, 2012). Lastly, Cav-1 silencing diminished IL-1beta-induced activation of p38-MAPK and MAPK-activated protein kinase 2 (MK2), as well as EC tube formation *in vitro* and angiogenesis *in vivo* (Jagielska *et al.*, 2012).

Delivery

Most standard transfection methods that yield sufficient *in vitro* transfection success with minimal toxicity cannot be applied for *in vivo* studies, e.g., due to immune response to viral vectors, toxicity of certain chemicals, or technical limitations as in electroporation. Poly(B-amino ester) polymers, known for their hydrolytic biodegradability, low toxicity, and triggered nucleic acid release, may be an alternative to commercial chemical agents (Arnold *et al.*, 2012). Complicating that, significant differences exist in the efficacy of transfection reagents and in the susceptibility of endothelial cells, smooth muscle cells as well as macrophages towards RNAi (Nabzdyk *et al.*, 2011; 2012).

The mannose receptor CD206 and macrophage/dendriteic cell marker was used to facilitate CD206-targeted RNAi delivery. Compared to control cells these nanoparticles delivered over 13-fold more siRNA into macrophages (Yu *et al.*, 2013).

VCAM-1 and E-Selectin are EC surface molecules upregulated in response to inflammation and play central roles in leukocyte adhesion and transmigration and thus contribute to the development of atherosclerosis. siRNA containing liposomal particles targeting VCAM-1 or E-Selectin have proven to selectively target inflamed endothelium, while not entering quiescent EC. This approach may prove successful for selective therapy of particularly active atherosclerotic lesions (Kowalski *et al.*, 2011; 2013; 2014; Leus *et al.*, 2014).

Local siRNA release from gelatin-PEI-siRNA coated vascular stents achieved significant silencing in EC (Nolte *et al.*, 2011). Similarly, PEI-siRNA complexes released from dip-coated electrospun polyethylene terephthalate bypass graft fabrics silenced target genes in infiltrating VSMC *in vitro* (Nabzdyk *et al.*, 2014).

Future studies will have to explore customizable delivery methods that allow for highly predictable cell/tissue specific delivery without causing significant off-target or immune responses. Anatomical barriers, diffusion distances, tissue specificity, lysosomal entrapment, controlled and prolonged release as well as hepatic and macrophage clearance are just some key aspects that need to be addressed in the coming years to improve *in vivo* siRNA therapies.

Discussion

Atherosclerosis and AA are complex, chronic diseases that involve a multitude of pathophysiologic processes, cell types, cytokines, and enzymes and thus a plethora of potential therapeutic targets exists. However, in many of the examples presented in this review, large animal *in vivo* target evaluation has to be performed to validate these targets for potential clinical trials. Bioinformatics and systems biology may help delineate these complex and often redundant cell signaling networks and identify crucial relay stations and regulators that could be targeted with siRNA.

While siRNA is a powerful technology, it also has significant limitations that need to be addressed in order to maximize effectiveness. siRNA molecules have a very short half-life *in vivo* and need to be chemically protected from thermal and enzymatic degradation. In addition, *in vivo* transfection efficiency is a vital determinant of target gene silencing. There is promising research in the field of cell specific siRNA delivery that may ultimately lead to the development of efficacious therapies (Kowalski *et al.*, 2011; 2013; 2014; Leus *et al.*, 2014; Leuschner *et al.*, 2011). This may decrease the amount of siRNA needed and mitigate unwanted off-target effects. siRNA off-target effects due to nonspecific siRNA/mRNA hybridization may incidentally adversely alter cell signaling.

Atherosclerosis is a systemic disease and chronic treatment using systemic administration of siRNA may neither be scientifically feasible nor cost effective. Additionally, the transient effect of siRNA on gene expression needs to be factored into the therapy. Whether repeated siRNA administrations are necessary or if the administration of a single bulk dose of siRNA for instance, encapsulated in nanoparticles in form of a depot is feasible, needs to be evaluated in future studies.

However, siRNA may soon become an adjuvant therapeutic for local application in diseased arterial segments, and/or in conjunction with surgical or interventional therapy. It is conceivable that in the near future siRNAs targeting specific genes may replace more toxic reagents such as sirolimus or tacrolimus in order to prevent restenosis after percutaneous angioplasty and stenting or mitigate intimal hyperplasia after arterial bypass grafting. In recent studies, vascular stents and bypass graft materials were successfully coated with siRNA. Large *in vivo* studies and clinical trials yet are still missing to validate these approaches (Nabzdyk *et al.*, 2014; Nolte *et al.*, 2011).

After the disappointing results of the PREVENT III trial there has been a concern regarding the usefulness of gene therapy for the treatment of vascular disease. With the advancements in bioinformatics, systems biology, and chemical engineering, gene therapy today is more sophisticated and customizable than ever before (Bhasin *et al.*, 2012; Kowalski *et al.*, 2014; Leuschner *et al.*, 2011). Such a customized interdisciplinary approach may render effective gene therapy for the treatment of atherosclerosis and AA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

siRNA Targets in Atherosclerosis.

Target Name	Mechanism	Study Type	References
IMMUNE MODULATOR			
Toll-like receptors 2 and 4 (TLR2 and 4)	inflammation modulation via nuclear factor-kappaB, chemokine secretion and MAPK signaling	*in vitro*	Ji *et al.*, 2011; Ji *et al.*, 2010; Geng *et al.*, 2010; Su *et al.*, 2011; Liu *et al.*, 2010; Meng *et al.*, 2013
Mammalian Target of Rapamycin (mTOR)	foam cell formation, LDL receptor, chemokine and MMP-2 expression	*in vitro* & mouse model	Yu *et al.*, 2011; Ma *et al.*, 2013a; Wang *et al.*, 2013b
C-C chemokine receptor type 2 CCR2	monocyte chemotaxis	mouse model	Leuschner *et al.*, 2011; Majmudar *et al.*, 2013)
Chemokine (C-X3-C motif) Receptor 1 (CX3CR1)	inflammation (TNF-alpha) and remodeling (MMP-9), EC to dendritic cell interaction	*in vitro*	Butoi *et al.*, 2011; Liu *et al.*, 2011
C-X-C chemokine receptor type 7 (CXCR7)	inflammation, chemotaxis (sdf-1)	*in vitro*	Ma *et al.*, 2013b)
Endoplasmic Reticulum (ER) Stress Transducers (protein kinase RNA-like endoplasmic reticulum kinase (PERK) and inositol-requiring protein 1 (IRE-1)	inflammation, leukocyte adhesion (ICAM-1, P-Selectin)	*in vitro*	Luo *et al.*, 2013; Yao *et al.*, 2013; Moreno *et al.*, 2013
S100 alarmin S100A9	neutrophil activity, macrophage vesicle calcification, SMC differentiation	*in vitro*	New *et al.*, 2013; Inaba *et al.*, 2009
LIPID METABOLISM			
Oxidized low-density lipoprotein receptor 1 (LOX-1)	oxLDL internalization and degradation	*in vitro*	Wang *et al.*, 2013a; Zhang *et al.*, 2013b
Apolipoprotein B (apoB)	lipoprotein shuttling	mouse model	Tadin-Strapps *et al.*, 2011)
REACTIVE OXYGEN SPECIES			
NADPH oxidase 1 (Nox1), NADPH oxidase, EF-hand calcium binding domain 5 (Nox5)	leukocyte adhesion, chemokine ligand expression	*in vitro*	Gray *et al.*, 2013; Bayat *et al.*, 2012; Rius *et al.*, 2013
TRANSCRIPTION FACTORS			
Nuclear factor-kappaB (NF-κB)	Il-17 induced VCAM expression in VSMC	*in vitro*	Zhang *et al.*, 2013a
Kruppel Like Factor 4 (KLF4)	SMC differentiation (Myocd)	*in vitro*	Turner *et al.*, 2013
MATRIX METALLOPROTEINASES (MMPs)			
Matrix metalloproteinase 2 and 9 (MMP −2/−9)	ECM remodeling, migration, apoptosis	*in vitro*	Johnson *et al.*, 2011; Kim *et al.*, 2012; Kowluru *et al.*, 2011
PROTEIN KINASES			
Extracellular signal-regulated kinases 1/2 (ERK1/2)	proinflammatory cytokines, cell adhesion molecules, oxLDL uptake, proliferation	*in vitro*	Li *et al.*, 2010
C-Jun	IL-6 and RANKL signaling	*in vitro*	Zhang *et al.*, 2012; Kim *et al.*, 2010
c-Jun NH2-terminal kinases (JNK, JNK2)	serum amyloid A signaling, EC alignment, NF-kB signaling, SMC migration and proliferation	*in vitro*	Dong *et al.*, 2011; Wang *et al.*, 2011; Hahn *et al.*, 2011; Wang *et al.*, 2011; Shyu *et al.*, 2012
p38 mitogen-activated protein kinase family (p38 MAPK)	LDL accumulation in macrophages, PPAR-gamma expression	*in vitro*	Mei *et al.*, 2012
Protein kinase C (PKC) - (PKCα/β/δ)	SMC adhesion, migration, CD36 expression, proinflammatory cytokines (IL-1, TNF-alpha, MCP-1, IL-6), apoptosis	*in vitro*	Jialal & Devaraj, 2012; Jialal *et al.*, 2013a; Jialal *et al.*, 2013b; Karunakaran *et*

Target Name	Mechanism	Study Type	References
			al., 2013; Larroque-Cardoso *et al.*, 2013
Janus kinase/Signal Transducer and Activator of Transcription (JAK/STAT)	cholesterol flux in macrophages	*in vitro*	Mo *et al.*, 2011; Park *et al.*, 2013b
CELL SURFACE MOLECULES/MEMBRANE RECEPTORS/MEMBRANE ASSOCIATED PRO TEINS			
Platelet Endothelial Cell Adhesion Molecule 1 (PECAM-1 or CD31) and Fibronectin (FN)	VCAM-1 expression, NF-kB signaling	*in vitro*	Wang *et al.*, 2011; Feaver *et al.*, 2010
Eph receptors Ephrin A2 (EphA2) and Ephrin A4 (EphA4)	VCAM-1 expression; monocyte adhesion, EC stress fiber formation	*in vitro*	Funk *et al.*, 2012; Jellinghaus *et al.*, 2013
Caveolin (Cav-1)	cholesterol flux in macrophages, macrophage differentiation. ICAM expression, proinflammatory cytokine (IL-1beta), EC tube formation	*in vitro*	Hu *et al.*, 2010; Fu *et al.*, 2012; Jagielska *et al.*, 2012

Table 2

siRNA Targets in Aortic Aneurysm.

Target Name	Mechanism	Study Type	Reference
IMMUNE MODULATOR			
Toll-like receptors 2 and 4 (TLR2 and 4)	vascular remodeling via MMP-2 and −9	*in vitro*	Cheng *et al.*, 2011
C-C chemokine receptor type 2 CCR2	monocyte chemotaxis	mouse model	de Waard *et al.*, 2010
S100 alarmin S100A4	SMC proliferation; remodeling via MMP	*in vitro*	Cao *et al.*, 2013
REACTIVE OXYGEN SPECIES			
NADPH oxidase 4 (Nox4)	adventitial fibroblast activation	*in vitro*	Liu *et al.*, 2012b
TRANSCRIPTION FACTORS			
Kruppel Like Factor 4 (KLF4)	SMC differentiation	*in vitro*	Salmon *et al.*, 2013
MATRIX METALLOPROTEINASES (MMPs)			
MMP-2	SMC invasion	*in vitro*	Kimura *et al.*, 2010
MMP−9/−12	SMC proliferation	*in vitro*	Dwivedi *et al.*, 2009
PROTEIN KINASES			
Extracellular signal-regulated kinases 1/2 (ERK1/2)	MMP-2 formation	*in vitro*	Ghosh *et al.*, 2012
c-Jun NH2-terminal kinases (JNK, JNK2)	MMP activity	*in vitro*	DiMusto *et al.*, 2012
Protein kinase B (AKT)	MMP−2/−9 expression and secretion	*in vitro*	Ghosh *et al.*, 2014