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REVIEW ARTICLE

Therapeutic implications of small interfering RNA in cardiovascular diseases

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ABSTRACT

Cardiovascular diseases (CVDs) place a heavy burden on the economies of low- and middle-income countries. Comprehensive action requires combining approaches that seek to reduce the risks throughout the entire population with strategies that target individuals at high risk or with established disease. Small interfering RNA (siRNA) as a functional mediator for regulation of gene expression has been evaluated for potential therapeutic targets for the treatment of various cardiovascular diseases such as hypertension, atherosclerosis, heart failure etc. The present review attempts have been made to provide a brief outline of the current understanding of the mechanism of RNAi and the delivery system and describe the therapeutic application of siRNAs and their potential for treating CVDs which are taking a heavy toll on human life.

INTRODUCTION

An estimated 17.1 million people die of cardiovascular diseases (CVDs) each year. According to WHO, CVDs are the number one causes of death globally. By 2030, almost 23.6 million people will die from CVDs, mainly from heart disease and stroke (http://www.who.int/mediacentre/factsheets/fs317/en/, accessed on 15 April 2012).

The occurrence of these CVDs has been on rise since decades. The major CVDs including hypertension, atherosclerosis, cardiac hypertrophy, and heart failure (HF) are highly prevalent but are still the 'untreated' disorders. For diseases like hypertension, atherosclerosis, and cardiac hypertrophy, there is no treatment because one can only control the disease and prevent the progression to a certain extent. Moreover, the drugs are associated with large number of side effects. For diseases like myocardial infarction and HF, surgery remains the only option. With the advancements in the biomedical technology, the understanding of the cellular and molecular mechanisms of CVDs has been on rise. Over the period of time, large numbers of molecular and genetic targets are identified. RNA interference (RNAi), a mechanism for RNA-guided regulation of gene expression, involves small interfering RNA (siRNA) as the functional mediator. Identification gene functions and evaluating their prospective therapeutic targets can utilize this constructive technology of RNAi [1]. Thus, prevention of and treatment for human disease such as cardiovascular diseases, neurological diseases, viral infections, cancer etc., are being targeted by huge numbers of biotechnological and pharmaceutical companies through development of RNAi-based drugs [2–4]. Thus, targeting pathogenic genes that are specific for CVDs, siRNA can offer greater advantage in terms of specificity, high potency, low toxicity, and complete remission of the disease. In the present review, we have given a brief over view of the mechanisms and delivery of siRNA and focused upon the therapeutic applications of this technology for various cardiovascular disorders.

MECHANISM OF siRNA AND ITS DESIGNING

The RNAi mechanism (*Figure 1*) involves two steps. The foremost step involves a Dicer or Dicer homologue that cleaves the double-stranded RNA (dsRNA) into 21- to 23-nucleotide-long fragments. Subsequently, the

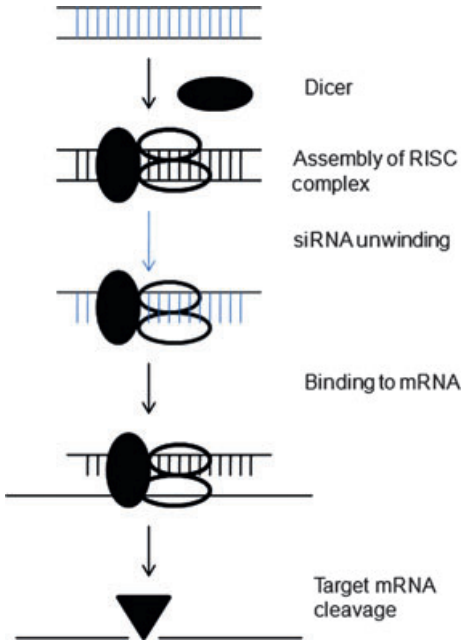

Figure 1 Mechanism of siRNA. RISC, RNA-induced silencing complex.

RNAi-specific enzymes possibly still including Dicer bind to the fragments (siRNA) generated by cleavage of dsRNA and get integrated into distinct nucleus complex, the 'RNA-induced silencing complex' (RISC), which targets the mRNA for degradation process. The siRNA couples with target mRNA and cleaves it from the centre of region recognized by siRNA. The passive siRNA strand not being used for the identification of target is displaced by ribonulcease temporarily. Constantly transcribing target mRNA can be degraded adequately for a long period of time by only few molecules of dsRNA. Although amplification of small siRNAs from long dsRNA can be achieved to some extent, it is inadequate to bring incessant degradation of mRNA. Therefore, second step involves amplification wherein long lasting post-transcriptional gene silencing is achieved by augmentation of dsRNA signal through RNA-dependent RNA polymerase (RdRp). In this manner, RdRp can convert an aberrant single-stranded RNA population into dsRNA, repetitively, duplicate dsRNA to yield a population of single-stranded RNA that could then interact with target RNA; or copy copies of the trigger thus generating a 'self-replicating' trigger population.

In the last few years, siRNAs have become the most sought after tool for RNAi as a treatment strategy for combating various diseases and disorders at the genetic level. The wide array of genomic sequence serves as the Bible for researchers to create siRNA design algorithms for effective and efficient identification of a gene for the purpose of inducing specific gene silencing. Effective siRNA designing becomes crucial to minimize costs and time constraints and maximize specificity. Several steps have been suggested for siRNA designing by Mysara et al. [5] during their trials to design a novel siRNA therapy. (*Figure 2*).

Various programs are now available for siRNA full automation designing which include MysiRNA-Designer, siDESIGN Center, Asi-Designer, RNAxs, siDRM [6]. Numerous factors are involved in siRNA designing process and due consideration must be given to them for achieving desired siRNA, which have been depicted in *Figure 3* with the sub-factors associated with them.

siRNA DELIVERY

siRNA delivery poses a greater challenge in its therapeutic implication. Naked siRNAs that are unprotected

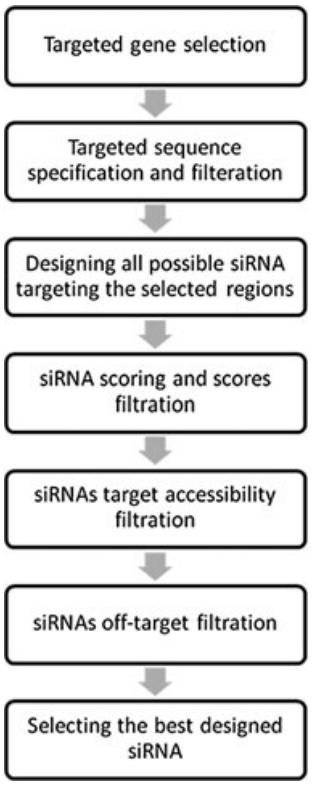

Figure 2 Steps for siRNA designing.

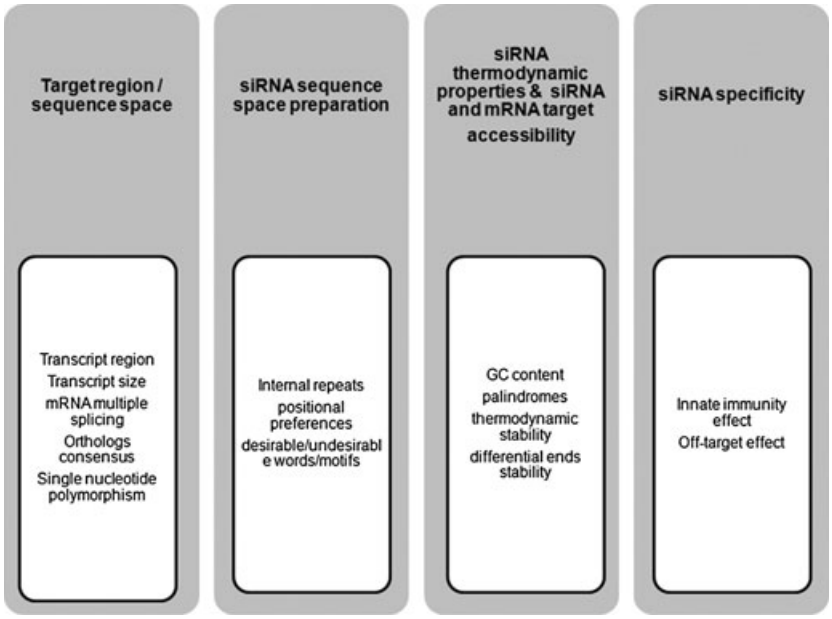

Figure 3 Factors involved in siRNA designing process.

and chemically unmodified face the threat of rapid degradation by serum RNAses. Moreover, siRNAs are negatively charged polymers and hence penetration through lipophilic cell membrane becomes almost impossible [7]. However, various approaches like chemical modifications, viral and non-viral nucleic acid deliveries etc., have been considered to eliminate the above-mentioned deleterious effects of siRNA delivery. siRNA are sensitive to nucleases, exhibit poor tissue distribution and poor membrane permeability. Hence, chemical modifications of this would help in the proper delivery of siRNA with increased half-life and functionality. Chemical modification of nucleobases, sugars and the phosphate ester backbone of siRNA can yield increased resistance to nucleases [8–10]. One approach involves induction of nucleus resistance by partial modification of phosphorothioate backbone and variation of 2′ O-methyl sugar on the sense and anti-sense strands. Alteration in tissue distribution was promoted by conjugation of cholesterol with 3′-end of the sense strand using a pyrroloidine linker [11]. Chemical modification of siRNA designed to augment its specificity resulted in the development of second generation siRNA [12,13].

Virus-mediated delivery methods are based on delivery of genes encoding short hairpin RNA (shRNA). Treatment for chronic diseases requires long-term RNAi for which viral expression vectors seem the most plausible tool. Encapsulation of siRNA involves utilization of reconstituted viral envelopes derived from influenza virus, which contains influenza virus spike protein hemagglutinin accountable for binding to and fusion with cellular membranes and also additionally added cationic lipids. These siRNA carrier vesicles are taken by receptor-mediated endocytosis which eventually escapes endosomal degradation. In vivo gene silencing by DNA-encoded shRNA delivery has been investigated to a larger extent. Various approaches for gene silencing through si/shRNA delivery involve the following: reporter gene expression inhibition in cortical neurons by intracranial delivery of lentivirus-produced shRNA [14], viral cyclin inhibition to check primary effusion lymphoma in mice by intraperitoneal delivery of lentivirus-produced shRNA [15], mutant SOD1 suppression in amyrotropic lateral sclerosis through intramuscular or intraspinal administration of lentivirus-produced shRNA [16,17] and inhibition of CC-chemokine receptor 2 in hematopoietic cells in mice by ex-vivo delivery of lentivirus-produced shRNA [18]. Newer approaches being studied these days include redirection of natural preferred cell type of viruses toward the surface receptors on target cell. However, the major drawback for both adenoviral-based and adeno-associated virus (AAV)-based vector is that strong immune responses are triggered on repeated administration, thereby limiting its application [19].

Delivery system based on RNA involves a system consisting entirely of RNA based on the packaging RNA of the DNA-packaging motor of bacteriophage phi29, which can spontaneously form dimmers via

interlocking right- and left-hand loops. Target cells can be transfected locally or systemically with complexes of siRNA and cationic polymers or lipids which are unshielded and untargeted for therapeutic effects. Target cell specificity can be achieved by cationic surface shielding that induces reduction in non-target tissue uptake and enhances the colloidal stability of the siRNA complexes. Cationic liposome seems to be promising for this purpose but is toxic. On the other hand, polyamines and peptides are less toxic, but they are difficult to attach to siRNA.

ROLE OF siRNA IN CVDS

Hypertension

Hypertension is a heterogeneous disorder, which remains a major modifiable risk factor for cardiovascular disease [20]. It is one of the most important risk factors for stroke, congestive HF, myocardial infarction, and peripheral vascular disease.

The renin angiotensin system has been an important regulator for blood pressure. Zhou et al. [21] investigated in spontaneous hypertensive rats (SHRs), the effects of RNAi targeting AT_1 receptor and ACE on blood pressure and myocardial remodeling. ACE, AT_1R, ACE and AT_1R gene-specific shRNA were expressed in SHRs by recombinant adenoviral vectors Ad5-ACE-shRNA, Ad5-AT_1R-shRNA, and Ad5-ACE-AT_1R-shRNA. A significant reduction in systolic blood pressure (SBP) along with antihypertensive effect lasting for at least 15 days was observed on injection with Ad5-ACE-shRNA, Ad5-AT1R-shRNA, and Ad5-ACE-AT1R-shRNA. Further SHRs treated with Ad5-ACE-shRNA, Ad5-ACE-AT1R-shRNA, Ad5-AT1R-shRNA, and Ad5-ACE-AT1R-shRNA exhibited a reduction in ACE and AT1 mRNA expression at ventricle and aorta with decrease in ratio of left ventricular to body weight (LVW/BW ratio) and myocardial collagen. Thus, prolonged antihypertensive effects and myocardial ultrastructural improvements owing to inhibition of inhibited myocardial and aortic ACE and AT_1R mRNA expressions could be observed in SHRs through RNAi targeting ACE and AT_1R gene [21]. In the rat, two distinct angiotensin II type 1a (rAT_{1a}) receptor mRNAs are synthesized from a single rAT_{1a} receptor gene by alternative splicing. These two transcripts are comprised of exons 1, 2, and 3 (E1,2,3) or exons 1 and 3 (E1,3). As exon 3 contains the entire coding region, both transcripts encode identical rAT_{1a} receptors [22,23]. Hassan et al. [24] aimed at using RNAi to selectively silence the rAT_{1a} receptor splice variants. A reduction in E1,3 mRNA and AT_1 receptor-specific binding was observed when rat aortic smooth muscle cells (RASMC) of male Sprague–Dawley rats was transfected with siRNA targeting E1,3. Further reduction in E1,2,3 mRNA and AT_1 receptor binding with no effect on E1,3 mRNA was recorded on treatment with E1,2,3-targeting siRNA ($S2_{E2}$). Thus, it was observed that E1,2,3 splice variant was responsible for majority of functional AT_1 receptor expression in RASMC and siRNA can be used to silence rAT_{1a} receptor mRNA in a splice variant-specific manner in RASMC. In another study, ACE silencing has been reported to exert significant anti-hypertensive effects in SHRs [25]. In this study, He et al. [25] treated SHRs with recombinant adenoviral vectors Ad5-EGFP-ACE-shRNA (EGFP is enhanced green fluorescent protein). A significant reduction in SBP along with decreased ACE mRNA expression in the myocardium, aorta, kidney, and lung was observed in ACE RNAi SHRs. RNAi also exhibited antihypertensive effect for more than 14 days suggesting RNAi against ACE as a target for treatment for hypertension.

Various tissues including heart, brain, lung, uterus, the gastrointestinal system, and vascular smooth muscle cells (VSMCs) express voltage-gated L-type calcium channel (Cav1.2) [26]. Isoform A of Cav1.2 is specifically expressed in the heart and isoform B, which is a non-cardiac form, expressed in various cell types including VSMCs [27]. Vascular Ca_L current and vascular tone is increased because of increase in different rodent models of hypertension attributed to the expression of the noncardiac form of the Ca_L ($Ca_v1.2$). So, Rhee et al. [28] designed a siRNA expression vector to knockdown the expression of the noncardiac form of Cav1.2 in VSMCs without affecting the cardiac Cav1.2 expression in the heart. Human embryonic kidney (HEK) 293 cells co-transfected with a rat $Ca_v1.2$ expression vector were initially screened with plasmids and appropriate controls. A cytomegalovirus promoter-driven modified mir-30a-based short hairpin RNA yielded the most effective gene silencing. It was found that cardiac L-Type calcium channel expression and function was unaffected by siRNA against Cav1.2 isoform B and effectively reduced vascular $Ca_v1.2$ expression. In a similar study, Pang et al. [29] injected the VSMC-specific siRNA expression cassettes into angiotensin II (Ang II)-induced hypertensive mice with the help of AAV vector. A reduction in blood pressure for nearly 4 weeks along with decrease in L-type Ca

currents from isolated mesenteric artery VSMCs was observed in AAV/Cav1.2 siRNA-treated mice. Thus, AAV-mediated VSMC-specific knockdown of $Ca_v1.2$ may provide a long-term therapy for hypertension. Thus, in vivo molecular intervention may prove to be a novel gene therapy for hypertension.

The sodium-selective amiloride-sensitive epithelial channel (ENaC) mediates electrogenic sodium re-absorption in tight epithelia and is deeply associated with human hypertension. The ENaC expression at plasma membrane requires the regulated transport, processing, and macromolecular assembly in a defined and highly compartmentalized manner. Ras-related Rab GTPases regulate intracellular trafficking during endocytosis, regulated exocytosis, and secretion [30–32]. To evaluate the role of these proteins in regulating amiloride-sensitive sodium channel activity, multiple Rab isoforms 3, 5, 6, and Rab27a were expressed in HT-29 cells by Saxena et al. [33]. HT-29 cells when initially were overexpressed with Rab isoforms resulted in inhibition of ENaC currents by Rab3 and Rab27a, whereas expression of other Rab isoforms failed to elicit any effect on ENaC currents. Further transfection of HT-29 cells with isoform-specific siRNA using lipofectamine HT-29 cells was cultured. The cells were transfected with Rab constructs using lipofectamine isoform-specific siRNA and they potentiated the amiloride-sensitive currents supporting the fact that Rab3 and Rab27a act as negative modulators of ENaC function. These observations point to the fact that Rab proteins in ENaC transport may serve as potential target for human hypertension.

The proinflammatory cytokine interleukin (IL) 6 has been reported to have role in cold-induced hypertension. Crosswhite and Sun [34] determined this in Sprague–Dawley rats which were given recombinant AAV carrying IL-6 shRNA on exposure to a cold environment (5 °C). It was observed that IL-6 shRNA controlled hypertension for $\geq$ 7 weeks with significant reduction in cold-induced elevation of SBP, IL-6 expression in the heart and decreased TNF-α expression in aorta. IL-6 shRNA delivery also led to abolition of glomerular collapses along with attenuation of cold-induced macrophage and T-cell infiltration in the kidney. Further RNAi knockdown of IL-6 prevented deposition of collagen around the coronary arteries and vascular superoxide production [34]. In addition to IL-6, IL-13 is also determined to be a regulator of pulmonary artery smooth muscles (PASMC) growth that governs idiopathic pulmonary hypertension (IPAH). Hecker et al. [35] reported that the exposure of primary human PASMC to IL-13 through expression of the *il13ra2* gene resulted in phosphorylation of STAT3 and STAT6 and reduction in PASMC cell growth by promoting G_0/G_1 arrest. Further IL-13ra2 knockdown by siRNA produced knockdown of il13ra2 mRNA levels and IL-13ra2 expression by 68% and 64% respectively, resulting in blunting of antiproliferative effects of IL-13 on PASMC [35].

Paulin et al. [36] described activation of the transcription factor nuclear factor of activated T-cells (NFAT) has major impact in human PAH and accounts for various features of PAH PASMC. Pim-1, a strong NFAT activator, is a kinase and has been thought to be expressed primarily in cancer cells. They demonstrated increased apoptosis, decreased NFAT activation, proliferation, pulmonary arterial pressure, wall thickness, and right ventricular hypertrophy using Pim-1 siRNA in rats with monocrotaline-induced PAH (M-PAH) and inhaled dominant-negative adenovirus (DN-Pim-1).Thus, Pim-1 can serve as a novel target treatment in human and experimental PAH.

Mitogen-activated protein kinase (MEK) pathway has been postulated to be involved in the process of cell proliferation of VSMC. An intravenous delivery system of siMEK using a novel nanoparticle technology was developed by Obata et al. [37]. siMEK/WL treatment along with inhibition of proliferation and apoptosis of VSMCs significantly attenuated increases in right ventricular systolic pressure and right ventricular weight-to-body weight ratio (RVW/BW ratio) in the rat model of PAH. Further inhibition of expression of MEK1/2 mRNAs in the lung and hypertrophy of the pulmonary arterial wall was observed because of selective accumulation of siMEK/WL in injured pulmonary arterial wall. Thus, inhibition of MEK by siMEK/WL may serve as a new therapeutic strategy for the treatment for PAH.

It has been found that Gq protein-coupled receptors (GqPCRs) serve as the major receptor for most vasoconstrictor systems. Wang et al. [38] targeted matrix metalloproteinase-7 (MMP-7) in rodent models of acute, long-term, and spontaneous hypertension by 3 complementary approaches. (i) They demonstrated MMP-7 as a mediator in acute hypertension (induced pharmacologically with adrenergic agonists (phenylephrine and norepinephrine), Ang II or *N*G-nitro-L-arginine methyl ester (L-NAME)) in normotensive Sprague–Dawley rats and C57BL/6 mice wherein co-administration of doxycycline resulted in reduction in MMP-7 activity. Further C57BL/6 mice were treated

with siRNA resulting in downregulation of MMP-7 activity in various tissues including aorta, heart, and small intestine. (ii) It was observed that MMP-$7^{-/-}$ resulted in attenuation of Ang II- and norepinephrine-induced rise in SBP. (iii) Further it was demonstrated that siRNA targeting of MMP-7 in SHRs resulted in significant reduction in SBP, MMP-7 activity in resistance arteries, attenuation of cardiac hypertrophy and inhibition of 'a disintegrin and metalloproteinases' (ADAM-12) transcription.

Manganese containing mitochondrial SOD (SOD2) present in mitochondria is a critical regulator in protection against excessive production of superoxide (O_2^-). Superoxide (O_2^-) has been implicated in the pathogenesis of many human diseases including hypertension. Dikalova et al. [39] investigated the role of mitochondria-targeted antioxidant mitoTEMPO wherein they treated Ang II-induced Bovine aortic endothelial cells (BAECs) with mitoTEMPO that resulted in increased mitochondrial O_2^- dismutation (threefold), complete blockage of increase in NADPH oxidase activity and recovery of endothelial NO. It was observed that similar effects were mimicked when Human aortic endothelial cells (HAECs) transfected with SOD2 plasmid resulted in 2.4-fold increase in mitochondrial SOD2 activity and HAECs transfected with siRNA recorded 2.7-fold reduction in SOD2 activity with increased NADPH oxidase. Further treatment for previously Ang II-induced hypertensive C57Bl/6 mice or mice transgenic for human SOD2 (tgSOD2 mice) with mitoTEMPO-attenuated hypertension and decreased blood pressure. Also transgenic mice overexpressing mitochondrial SOD_2 demonstrated attenuated Ang II-induced hypertension and vascular oxidative stress similar to mice treated with mitoTEMPO [39].

Pulmonary and systemic arterial hypertension are associated with profound alterations in Ca^{2+} homeostasis and smooth muscle cell (SMC) proliferation. A novel class of non-selective cation channels, the transient receptor potential (TRP) channels, has emerged at the forefront of research into hypertensive disease states. TRP channels have been identified as molecular correlates for receptor-operated and store-operated cation channels in the vasculature. TRP channels are implicated as Ca^{2+} entry pathways in pulmonary hypertension and essential hypertension [40]. Liu et al. [41] initially evaluated the functional aspect of increased TRPC3 channel expression in SHR wherein a significant increase in calcium was recorded in monocytes from SHR compared with normotensive Wistar–Kyoto rats (WKY). Further they demonstrated that the calcium increase in monocytes was significantly reduced in SHR and WKY in the presence of the TRPC channel blocker SKF-96365. These observations revealed that TRPC3 expression plays an important role in the pathogenesis of hypertension. They also carried out the transfection of monocytes with siRNA specific for TRPC3 for 24 h. TRPC3 knockdown because of RNAi resulted in significant reduction in increased cytosolic calcium on administration of external calcium in SHR suggesting it to be a novel target for the treatment for hypertension.

The role of arylsulfatase B (ASB) in the regulation of the kininogen–bradykinin axis owing to its effect on chondroitin-4-sulfation (C4S) and the interaction of C4S with kininogen has been reported by Bhattacharyya et al. [42]. It was reported that silencing or overexpression of ASB in normal rat kidney epithelial cells in tissue culture modified the content of total sulfated glycosaminoglycans, C4S, kininogen, and bradykinin in spent media and cell lysates. Overexpression of ASB resulted in decline of C4S that suggested vital role for C4S in regulating the kininogen C4S interaction. These findings suggest that ASB, owing to its effect on C4S, may impact on the kininogen–bradykinin axis and thereby, ASB activity may serve as a link between salt responsiveness and the bradykinin-associated mechanism of blood pressure regulation [42]. Zhang et al. [43] employed RNAi specific to phosphoinositide-3-kinase, the regulatory subunit 1 (PIK3R1) to investigate its inhibitive effects on the activity of splenic macrophage (MΦ) associated with hypersplenism because of portal hypertension (HS-PHT). Plasmid vector pGenesil-1 expressing specific shRNA against PIK3R1 and the scrambled shRNA control was constructed and transfected into HS-PHT-MΦ. They found that the knocking down of PIK3R1 with shRNA produced by pGenesil-1 led to inhibition of viability and decreased activity of MΦ associated with HS-PHT in vitro. *Table I* summarizes application and target genes of siRNA for hypertension.

ATHEROSCLEROSIS

Atherosclerosis is the principal cause of heart attack, stroke and gangrene of the extremities. The lesions result from an excessive, inflammatory-fibroproliferative response to various forms of insult to the endothelium and smooth muscle of the artery wall [44]. Vascular cell adhesion molecule-1 (VCAM-1), which is a

Table I Summary of the siRNA target genes for hypertension.

Target	Model / Process	Effect	References
IL-6	Cold induced HT	↓ SBP	[34]
IL-13		↓ Proliferation of PASMC	[35]
MEK	Rat model of PAH	↓ Right ventricular systolic pressure, ↓ right ventricular weight to body ratio	[37]
Pim-1	PAH-PASMC	↑ Apoptosis, ↓ proliferation, ↓ pulmonary arterial pressure, wall thickness and right ventricular hypertrophy	[36]
AT1 receptor and ACE	SHRs	↓SBP, ACE & AT1 mRNA expression	[21]
rAT1a receptor splice variants	RASMC	Reduction in E 1,3 mRNA & ↓in AT1 receptor binding	[24]
ACE	SHRs	↓ SBP, ↓ serum concentration	[25]
Cav1.2	Ang II-induced hypertensive mice	↓ BP, ↓ L-type Ca current	[29]
MnSOD	Ang II-induced HT	↓HT, ↓ BP	[39]
Rab isoforms 3,5,6 & Rab 27a	HT-29 cells		[33]
L-Type Ca channel	HEK 293 cells	↓ Cav1.2 expression	[28]
TRPC 3	SHR monocytes	↓TRPC3 expression, ↓ Ca^{+2} influx, ↓thapsigargin induced sustained Ca^{+2} ↑	[41]
ASB	Normal rat kidney epithelial cells	↓ C4S	[42]
PIK3R1	HS-PHT-Mϕ	↓ Activity of Mϕ, inhibition of viability	[43]
MMP-7	Rodent models of acute, long-term and spontaneous hypertension	Attenuation of HT, stopped development of cardiac hypertrophy	[38]

IL, interleukin; HT, hypertension; PASMC, pulmonary artery smooth muscle cells; MEK, Mitogen-activated protein kinase; PAH, pulmonary arterial hypertension; AT_1 receptor, angiotensin II type 1 receptor; ACE, angiotensin converting enzyme; SHRs, spontaneous hypertensive rats; SBP, systemic blood pressure; mRNA, icro-ribonucleic acid; RASMC, rat aortic smooth muscle cells; Ang II, angiotensin II; BP, blood pressure; PAI-1, plasminogen activator inhibitor-1; MnSOD, mitochondrial superoxide dismutase; HEK, human embryonic kidney; TRPC3, transient receptor potential canonical channel 3; Ca^{+2}, calcium; ASB, arylsulfatase B; C4S, chondroitin-4-sulfation; PIK3R1, phosphoinositide-3-kinase, the regulatory subunit 1; HS-PHT-Mϕ, hypersplenism because of portal hypertension of splenic macrophage; MMP-7, matrix metalloproteinase-7; ↓, decrease; ↑, increase.

member of the immunoglobulin super family, is abundantly expressed by SMC in atherosclerotic lesions and in injured arteries.

Petersen et al. [45] examined the role of VCAM-1 in SMC migration. SMCs were isolated from the descending thoracic aorta of female C57BL/6 mice and subsequently transfected with siRNAs targeting VCAM-1. Inhibition of VAM-1 expression by SMC at transcript and protein level was observed with reduction in the number of migrated cells into the scratch area. Also slight inhibition of proliferation of cells was observed. Thus, targeting VCAm-1 by siRNA technology can prove to be a treatment option for atherosclerosis.

It has been demonstrated by Sadahiro et al. [46] that the intercellular adhesion molecule-1 (ICAM-1) and VCAM-1 located play greater role as adhesion molecules during endothelial activation especially within the microvascular structures of the graft. The expression of adhesion molecules leads to tethering, rolling, sticking, and transendothelial migration of leukocytes, followed by release of proinflammatory factors in the interstitial compartment [47] and eventually leading to atherosclerotic alterations. These cascade-like reactions are mainly triggered by adhesion molecules including ICAM-1 and VCAM-1. Thus, suppression of these adhesion molecules may serve as a promising therapeutic option to reduce CVS complications. Walker et al. [48] tried to quantify at to what extent cardiac microvascular structures express the adhesion molecules E-selectin (ESELE), ICAM-1, and VCAM-1 and how specific siRNA targeting them interrupt pathophysiological processes. They transfected Human cardiac microvascular endothelial cells (HCMECs) with specific siRNA targeting ESELE, ICAM-1, and VCAM-1 which were further stimulated with tumor necrosis factor-α (TNF-α). It was found that stimulation of HCMECs with TNF-α resulted in a significant increase in receptor expression of all three adhesion proteins. A significant reduction in expression of adhesion molecules at both mRNA and protein level was found on transfection of microvascular endothelial cells with specific siRNA against ESELE, ICAM-1, and VCAM-1 followed by TNF-α stimulation. Also silencing through siRNA led to reduction in adhering leukocytes. In another study Walker et al. [49] transfected Primary human venous endothelial cells(HVECs) with siRNA targeting specifically ICAM-1,

VCAM-1, and ESELE using liposomal solution followed by stimulation with TNF-α. In this case, also a significant reduction in surface expression of these adhesion molecules and granulocyte adhesion was observed. Thus, induction of siRNA therapy against the adhesion molecules might prove beneficial for the reduction in inflammatory reactions and subsequent atherosclerotic development after graft surgery.

C-reactive protein (CRP), which is a member of the pentraxin family, circulates as a pentamer (pentameric CRP) in plasma and is a predictor of cardiovascular risk like coronary heart disease, ischemic stroke, and vascular mortality [50]. It is also a predictor of coronary events and may have direct actions on the vessel wall in the evolution of atherosclerosis. Potential wider applications include other inflammatory, infective, and tissue-damaging conditions characterized by increased CRP production, in which binding of CRP to exposed ligands in damaged cells may lead to complement-mediated exacerbation of tissue injury [51]. Endothelial cells release small vesicles known as Endothelial microparticles (EMPs) in response to activation or apoptosis by various stimuli [52]. Circulating endothelial cells (CECs) are circulating bodies present in blood owing to the shedding of damaged intimal endothelial layer [53]. This physical detachment of cells from the vessel wall may be due to initiation of proapoptotic signaling cascades. Devaraj et al. [54] have shown for the first time that increased release of CECs and EMPs is promoted by CRP, both in vivo and in vitro from HAEC. Thus, their data not only supports the fact that CRP induces endothelial dysfunction but also reveals that CECs and EMPs are important biomarkers of endothelial dysfunction in various CVDs including atherosclerosis. Receptor for advanced glycation end products (RAGE) is probable to accelerate the processes of atherosclerosis through inflammatory processes and endothelial activation. Chen et al. [55] cultured the endothelial progenitor cells (EPCs) isolated from bone marrow of Sprague–Dawley rats in presence and absence of CRP and evaluated the RAGE protein expression and apoptosis. It was observed that CRP-induced upregulation of both RAGE protein and RAGE mRNA level in ECPs, increased ROS production, decreased SOD and glutathione peroxidase (GSH-PX) mRNA levels and induced apoptosis. These effects were attenuated when RAGE protein expression was blocked by siRNA supporting the fact that CRPs upregulate RAGE expression along with ROS production and decreased antioxidant enzymes, thereby having direct role in the progression of atherosclerosis.

Although accumulation of VSMCs in the intima is a key event in the development of arterial lesions, apoptosis of VSMCs also plays an important role in progression of atherosclerotic lesions and contributes to increased plaque vulnerability. Blaschke et al. [56] demonstrated that CRP induces caspase-mediated apoptosis of human coronary VSMCs. GADD153 is a gene involved in growth arrest and apoptosis in vascular and nonvascular cells which is regulated at both transcriptional and posttranscriptional levels. CRP regulation of GADD153 mRNA expression in VSMCs occurs primarily at the posttranscriptional level by mRNA stabilization. Primary human coronary artery VSMCs was transfected with siRNA to inhibit GADD153 expression. A significant reduction in CRP-induced GADD153 mRNA expression (46.7%) and CRP-induced apoptosis (36.4%) was observed by GADD153-specific siRNA.

Adiponectin is an adipocytokine secreted by adipocytes, having antiatherogenic effect and inhibits inflammation. Kobashi et al. [57] explored the effect of adiponectin on endothelial synthesis of IL-8 which has a major role in atherogenesis. Inhibition of Il-8 secretion and IL-8 mRNA expression was observed when HAECs stimulated with TNF-α were treated with adinopectin. They also showed that Akt phosphorylation is enhanced by adinopectin. Pretreatment of TNF-α stimulated HAECs with Akt siRNA showed inhibition of adinopectin effect on TNF-α-induced IL-8 synthesis, which emphasized on the fact that adinopectin action on inhibition of IL-8 synthesis may be mediated by Akt activation. Thus, adinopectin may serve as a future target for atherosclerotic treatment by inhibition of NF-κB and activation of Akt phosphorylation.

The leukocyte-type 12/15-Lipoxygenase (12/15-LO) enzyme and its oxidized lipid products play important roles in VSMC growth, migration, and matrix responses associated with hypertension, atherosclerosis, and restenosis [58,59]. Dwarkanath et al. [61] showed that expression of chemokine monocyte chemoattractant protein-1 (MCP-1) can be upregulated in VSMC by 12/15-LO. They reported a reduction in MCP-1 mRNA and NF-κB expression in 12/15-LO knockout mice. It was also observed that MCP-1 gene expression increased when 12/15 LO was overexpressed. Further they transfected human VSMC with p65 siRNA directed against p65 subunit of NF-κB wherein it was observed that TNF-α induced MCP-1 gene expression decreased by 85%. These human VSMCs also exhibited blocked expression of MCP-1 and TNF-α by siRNA.

Thus, NF-κB could prove to be a novel target by RNAi in atherogenic conditions [60].

Omi/HtrA2 is a proapoptotic mitochondrial serine protease that is released into the cytosol during apoptosis and promotes cytochrome *c* (Cyt *c*)-dependent caspase activation by neutralizing inhibitor of apoptosis proteins (IAPs) via its IAP-binding motif. The protease activity of Omi/HtrA2 also contributes to the progression of both apoptosis- and caspase-independent cell death [61]. Martins et al. [62] found that over expression of Omi/HtrA2 sensitizes cells to apoptosis, and its removal by RNA interference reduces cell death. U2OS cells were transfected with Omi/HtrA2 siRNA that led to the elimination of Omi/HtrA2 expression and considerable reduction in the level of cell death. Thus, knockdown of Omi/HtrA2 renders cell more resistant to induction of apoptosis, and this could serve as target in slowing the progression of atherosclerosis. Caveolin-1 (Cav-1) a membrane protein can positively or negatively influence the advancement of atherosclerosis. Hu et al. [63] reported overexpression of PPAR γ1 and Cav-1 with increase in cholesterol efflux and attenuation of atherosclerotic lesions when murine macrophage cell line RAW264.7 was treated with AdPPARγ1. Further transfection with Cav-1-siRNA resulted in decrease in cholesterol efflux in RAW264.7 cells. Thus, PPAR γ1-enhanced Cav-1 can be a major target in attenuation of atherosclerosis.

Atherosclerosis and arterial interventions induce oxidative stress mediated in part by NADPH oxidases that have an essential role in the progress of neointimal hyperplasia and restenosis. Li et al. [64] targeted the NOX2 component of NADPH oxidase in arterial wall of atherosclerotic rat model by transfection of siRNA using amino-acid-based nanoparticle HB-OLD7. They observed a decline in Cybb gene expression with reduced neointima-to-media-area ratio and increased lumen-to-whole-artery area ratio. Thus, Cybb gene can be a functional target to prevent neointimal hyperplasia and atherosclerosis. A summary of target genes of siRNA for atherosclerosis is given in *Table II*.

STROKE

Stroke is a medical emergency because of blockage of blood flow to the brain resulting in deficiency of blood and oxygen to the brain cells ultimately leading to death. Clogging of arteries leads to ischemic stroke, and hemorrhagic stroke is a result of bursting of the blood vessel in brain (http://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0001740/, accessed on 15 April 2012.).

Neurons and astrocytes respond differently to ischemic stress, and their interaction becomes of great importance in various neurodegenerative pathologies where astrocytes have many functions that support the viability of neurons such as buffering the extracellular

Table II Summary of the siRNA target genes for atherosclerosis.

Target	Model / Process	Effect	Reference
VCAM-1	SMC from aorta of C57BL/6 mice	↓ No. of migrated cells	[45]
ESELE, ICAM-1 and VCAM-1	HCMECs	↓ Expression of ESELE, ICAM-1 and VCAM-1, ↓ no. of adhering leukocytes	[48]
ESELE, ICAM-1 and VCAM-1	HVECs	↓ Granulocyte adhesion	[49]
RAGE protein	EPCs	↓ RAGE in ECPs, ↑ ROS production and↓ apoptosis	[55]
GADD 153 gene	Human coronary VSMCs	↓ CRP induced apoptosis	[56]
NF-κβ Akt phosphorylation	HAEC		[57]
NK-κB	Human VSMC	↓ p65 protein level, blocked 13-HPODE-induced expression of MCP-1 and TNF-α	[60]
Omi/HtrA2	-	Reduced cell death	[62]
Cav-1	RAW264.7, PPARγ1-treated apoE-deficient mice	Induce Cav-1 expression, ↑ cholesterol efflux, attenuate atherosclerosis in apoE-deficient mice	[63]
NOX-2	Atherosclerotic rat model	↓ Cybb gene expression and neointima-to-media ratio, ↓ lumen-to-whole –artery ratio	[64]

SMC, smooth muscle cells; VCAM-1, Vascular cell adhesion molecule-1; HCMECs, human cardiac microvascular endothelial cells; ICAM-1, intercellular adhesion molecule-1; ESELE, E-selectin; HVECs, Primary human venous endothelial cells; ECPs, endothelial progenitor cells; VSMCs, vascular smooth muscle cells; CRP, C-reactive protein; NF-κB, nuclear factor κB; HAEC, human aortic endothelial cells; Cav-1, Caveolin-1; PPARγ, peroxisome proliferated γ; ↓, decrease; ↑, increase.

space and providing substrates to neurons [65]. In neurons, Transglutaminase 2 (TG2) has been found to be defensive against ischemic cell death both in vitro and in vivo [66]. Colak et al. [67] used a complete embryonic TG2 knockout (TG2−/−) mouse to study the effect of TG2 ablation in stroke injury. They observed that the stroke volumes in the TG2−/− mice were significantly smaller (~36%) than in wild-type mice. Also TG2−/− mouse primary neurons showed significantly lower (~12%) viability after oxygen–glucose deprivation (OGD) suggesting that TG2 ablation in neurons potentiates OGD-induced cell death. But at the same time, TG2−/− mouse astrocytes showed significantly greater viability (~25%) compared to wild-type astrocytes after the treatment suggesting that TG2 ablation in astrocytes protects them against OGD-induced cell death. These results suggest that in the TG2−/− mice, the reduction in stroke volume might be due in part to the increased survival of TG2−/− astrocytes and thus targeting TG2 may prove to be beneficial in stroke treatment therapy.

Caspase-3 has a major role in regulation of cell death and thus serves as a prime target for neuroprotective treatments [68]. Caspase-3 activation has a significant contribution in brain tissue loss after traumatic brain injury [69]. Disruption of caspases genetically after stroke can reduce ischemic neuronal injury inducing a neuroprotective effect [70]. Al-Jamal et al. [71] used an endothelin-1-induced stroke model and transfected the neurons with caspase-3 siRNA (siCas 3) delivered by functionalized carbon nanotubes (f-CNT) to observe the neuroprotective effect on downregulation of caspase pathway. Evaluation of neuroprotective mechanism was measured by performing the functional skilled reaching test in rats. The capability of f-CNT:siCas 3 treated group after induction of ischemic damage to reclaim food pellets served as a parameter for the assessment of functional effects. Thus, Jamal et al. [71] demonstrated that in vivo gene silencing of caspase-3 in neuronal tissue can lead to neuroprotection and considerable functional improvement before and after ischemic insult and could be a potential target for treatment for stroke.

The blood brain-barrier (BBB) is the brain's most crucial covering against entry of various cells and soluble factors from blood to brain [72]. The MMP plays an important role in disruption of BBB during various inflammatory conditions like multiple sclerosis, encephalitis, and cerebral ischemia. MMPs are proteolytic enzymes which are produced on stimulation by neurons, leukocytes, glial cells, and endothelial cells [73] that are involved in the turnover of the extracellular matrix (ECM) and are mediators of cell migration [74]. It has been found that MMP-9 mediates the transmigration of inflammatory leukocytes across basement membranes, thus leading to proteolytic degradation of ECM components. Tissue inhibitor of metalloproteinase-1 (TIMP-1) binds to both the active and precursor form of MMP-9 and inhibits its enzymatic activity. Collagens are also essential for the maintenance of the endothelial permeability barrier. Bonoiu et al. [75] used a well-validated in vitro brain model consisting of primary cultures of human brain microvascular endothelial cells (BMVEC) and normal human astrocytes (NHA) which were transfected with quantum dot (QD) complexed with MMP-9-siRNA (nanoplex) to downregulate the expression of MMP-9 gene in BMVEC. A significant decrease in the expression of the MMP-9 gene (78%) accompanied by subsequent reduction in biological activity of MMP-9 (56%) was observed. Along with this an increase in TIMP gene expression and increase in expression of collagen Iα1, collagen IV, and collagen V genes were observed. Thus, MPP-9 that has a key role in disruption of BBB during neuroinflammation can be silenced using siRNA technology and help in preserving BBB functionality in neurological conditions like cerebral ischemia. Hence, MMP-9 could serve as a future target in stroke treatment.

Inflammatory damage is induced by adhesion of leukocytes to the activated endothelium followed by brain invasion after cerebral ischemia. The invasion by lymphocytes largely depends on the interaction of the leukocyte very late antigen-4 (VLA-4) with VCAM-1 on endothelial cells [76]. Liesz et al. [77] examined the inhibition of VLA-4–VCAM-1 axis on secondary tissue injury after experimental brain ischemia. In vivo silencing of VCAM-1 was induced through siRNA transfection into the femoral vein. A significant reduction in infarct volume and cerebral leuckocyte migration was observed 6 days after middle cerebral artery occlusion (MCAO) through VCAM-1 silencing. Further this suggested that VCAM-1 is the main complementary endothelial ligand for post-ischemic leukocyte migration via VLA-4. Thus, inhibition of cerebral lymphocyte migration may prove to be a promising new approach for ischemic stroke.

Mesenchymal stem cells (MSCs) have the ability to differentiate and aggregate at the site of tissue/organ

Table III Summary of the siRNA target genes for stroke.

Target	Model / Process	Effect	Reference
TG2	TG2−/− mouse	↓ stroke volume, ↓ primary neurons viability, ↑ astrocyte viability	[67]
caspase-3	Endothelin-1 induced stroke model	Functional ability to retrieve food pellets retained	[70]
MMP-9	BMVEC and NHA	↓ MMP-9 gene expression,↑ in TIMP, collagen Iα1, collagen IV and collagen V gene expression	[75]
VCAM-1		↓ Infarct volume, ↓cerebral leukocyte migration	[77]
CXCR4	rMSCs	↓ Infarct volume, ↑ SDF-1, ↑ mobilization of rMSCs	[81]
HMGB1 gene	Primary cortical cultures	↓ Infarct volume	[84]

TG2−/−, TG2 knockout; MMP-9, matrix metalloproteinase; BMVEC, brain microvascular endothelial cells; NHA, normal human astrocytes; rMSCs, rat mesenchymal stem cells; SDF-1, stromal cell-derived factor-1; HMGB1, high mobility group box-1.

damage [78] and induce recovery of neurological function in cerebral infarction [79]. Stromal cell-derived factor-1 (SDF-1) and its receptor, CXCR4, have major role in stem cell homing, chemotaxis, modulating the expression of adhesion molecules, engraftment, proliferation, and cell survival [80]. Yu et al. [81] overexpressed the CXCR4-eGFP fusion protein (CXCR4/eGFP) by siRNA transfection using lentiviral vectors in the rat mesenchymal stem cells (rMSCs). It was observed that CXCR4 expression was significantly higher in the CXCR4/eGFP-rMSCs group. A considerable reduction in infarct volume was also observed in the CXCR4-rMSC-treated group. Upregulation of SDF-1 protein was observed 7 days after MCAO in the injured hemisphere of PBS-treated rats, especially in the penumbral regions. Thus, they concluded that cell repair mechanism and angiogenesis in the penumbral region was because of CXCR4-induced augmentation of rMSC mobilization into the ischemic areas of the brain. Thus, CXCR4 can serve as a treatment opportunity for decline in infarct volume and improved recovery in rats with cerebral ischemia.

Highmobility group box-1 (HMGB1 gene) has been found to function as a damage-associated molecular pattern (DAMPS) and as an alarmin, an endogenous molecule that signals tissue and cell damage [82]. HMGB1 serves as a danger signal when released extracellularly that evokes inflammatory reactions by activating various types of cells, especially immune-related cells [83]. Kim et al. [84] transfected primary cortical cultures and in rat brain with siRNA against HMGB1 delivered by e-PAM-R. A significant reduction in infarct formation was revealed by staining of both cortical and striatal region. Total infarction volume was reduced to 70.6% in ischemic hemisphere by HMGB1 siRNA/e-PAM-R complex. Thus, siRNA-mediated knockdown of HMGB1 can prove beneficial in stroke treatment. A summary of target genes of siRNA for atherosclerosis is given in *Table III*.

HYPERTROPHY

Cardiac hypertrophy is a compensatory response to a variety of physiological or pathological stimuli. However, prolonged hypertrophic responses may eventually lead to HF, arrhythmia, and sudden death. A number of intracellular signaling pathways have been implicated to play a critical role in the regulation of cardiac hypertrophy. Cyclic nucleotide phosphodiesterases (PDEs) through degradation of cAMP and/or cGMP regulate the amplitude and duration of intracellular cyclic nucleotide signaling. Miller et al. [85] found that phenylephrine-induced myocytes hypertrophy and hypertrophic marker expression in neonatal rat ventricular myocytes (NRVMs) and adult rat ventricular myocytes can be prevented by transfection of siRNA against phosphodiesterase 1A (PDE1A). They observed a decreased PDE1A protein synthesis and reduced total myocytes surface area. Thus, PDE1A may prove to be novel target for mediating the antihypertrophic effect of PDE1 inhibition in NRVMs.

Cardiac remodeling is associated with hypertrophy and fibrosis processes, which may depend on the activity of MMPs and ADAMs. Wang et al. [86] targeted tumor necrosis factor-α-converting enzyme (TACE) also known as ADAM-17 in rodent models of spontaneous and agonist-induced hypertension using RNAi. SHRs were infused with previously validated TACE siRNA through subcutaneously implanted ALZET osmotic minipumps in the back of the animals. This resulted in systemic knockdown of TACE expression and decreased the cross-sectional width of cardiomyocytes by 38%

which successfully stopped the advance of cardiac hypertrophy evidenced by M-mode echocardiography and gross pathology studies. Moreover, decline in Ang II-induced over expression of markers of myocardial hypertrophy and fibrosis, as well as ADAM-12 and MMP-2, was reported. Thus, TACE can prove to be an efficient target for hypertrophy treatment.

Calcium influx through the L-type calcium channels regulates calcium cycling in heart. Calcium cycling figures prominently in excitation-contraction coupling and in various signaling cascades involved in the development of left ventricular hypertrophy. Cingolani et al. [87] hypothesized that repression of cardiac hypetrophy and calcium current modulation can be induced by suppressing β-subunit of L-type calcium channel at genetic level. Neonatal rat cardiac myocytes (NRCMs) was transfected with shRNA template using lentiviral vector (PPT.CG.H1.β_2) directed against L-type calcium channel accessory β-subunit gene. They reported a decline of hypertrophic response with reduction in left ventricular wall thickness (33%) and heart weight/body weight ratios in PPT.CG.H1.β_2-injected rats without compromising systolic performance. Further a significant decrease in L-type calcium current was also observed. Thus, calcium channel β subunit can be targeted as a therapeutic strategy for left ventricular hypertrophy.

Calcineurin, which binds to the Z-disc in cardiomyocytes via α-actinin, promotes cardiac hypertrophy in response to numerous pathologic stimuli [88]. Li et al. [89] demonstrated interaction of muscle-specific F-box protein called atrogin-1, or muscle atrophy F-box with calcineurin A and α-actinin-2 at the Z-disc of cardiomyocytes. They observed an enhancement of calcineurin activity, cardiomyocyte hypertrophy and cardiomyocyte surface area in response to downregulation of atrogin-1 using adenoviral siRNA. In contrast to this, they reported a decline in heart weight/body weight ratios (25%) and collagen deposition with thinning of septum walls and left ventricular chambers in transgenic mice with overexpressed atrogin-1. Thus, calcineurin can serve as a target for blunting the progress of cardiac hypertrophy.

Evidence from in vivo studies suggests that some inputs to cardiac hypertrophy are opposed by the actions of oestrogen. Pedram et al. [90] showed that AngII- or endothelin-1 (ET-1)-induced new protein synthesis, skeletal muscle actin expression, and increased surface area in cultured NRCMs can be prevented by 17β-estradiol (E_2). Inhibition of AngII-induced calcineurin activity and decreased protein synthesis by E_2 was considerably reduced by transfection with siRNA against MCIP1 gene. Thus, 17β-estradiol can prove to be a better target for hypertrophy prevention.

Apart from cardiac hypertrophy, siRNAs have been also explored for VSMC proliferation and hypertrophy. Anomalous coronary vascular smooth muscle cell (CSMC) proliferation is a critical event underlying intimal hyperplasia, which eventually leads to hypertension. Dapas et al. [91] determined the effect of siRNA transfection against E2F1, cyclin E1, and cyclin E2 genes on human CSMC growth decline. They observed a decrease in mRNA and protein level for E2F1, cyclin E1, and cyclin E2. Further reduction of 57%, 84%, and 83% in CSMC number was reported by siRNA transfection directed against E2F1, cyclin E1, and cyclin E2, respectively. Decline in cell proliferation and migration of CSMC was also reported along with reduction in amount of S-phase cells and rise in G1-G0–phase cells. They also demonstrated a close control in regulation of all three genes. Thus, E2F1–cyclin E1–cyclin E2 can be targeted for hypertrophy treatment.

Kouri et al. [92] evaluated the plasminogen activator inhibitor (PAI)-1 which is a target gene for transforming growth factor (TGF)-β1 signaling cascade and an inhibitor of the plasminogen activator system for its function and expression. PAI-1 function was determined using active recombinant (r) PAI-1 or PAI-1-specific siRNA transfection in primary PASMCs. It was observed that siRNA-mediated PAI-1 knockdown resulted in decline of PASMCs migration and amplification of its proliferation, whereas rPAI-1-induced stimulation led to decreased PASMCs proliferation and adhesion to vitronectin and increased PASMCs migration [92].

HEART FAILURE

Heart failure is a chronic, long-term condition in which the heart can no longer pump enough blood to the rest of the body [93]. Phosphalamban (PLB) which is a muscle-specific protein has a key role to play in controlling the cardiac contractility. Phosphorylated PLB releases the inhibition of sarcoplasmic reticulum Ca2_ ATPase pump (SERCA2a) resulting in increased calcium influx into the sarcoplasmic reticulum and enhancing the contractility of cardiac muscle. AAV vectors have been found to exhibit expression for long periods of time in the heart [94,95] and can infect both dividing and non-dividing cells.

Reduced SERCA2a expression and/or PLB phosphorylation is the cause of dysfunctioning of PLB SERCA2a leading to malfunctioning of the failing heart. Successful treatment of HF was demonstrated in a rat model of transaortic banding by RNAi targeting of PLB, a key regulator of cardiac Ca^{2+} homeostasis [96]. Suckau et al. [96] demonstrated an improvement of hemodynamics in HF rats by aortic root injection through PLB silencing in the cardiomyocytes primary NRCMs by adenoviral shRNA vector (AdV-shRNA). A dimeric cardiotropic AAV vector (rAAV9-shPLB) was developed for long-term therapy to transfect NRCMs for induction of RNAi activity to the heart via intravenous injection. A reduction in Cardiac PLB protein and inhibition of SERCA2a was inhibited in HF groups. Restoration of diastolic and systolic functional parameters to normal ranges along with reduction in left ventricular (LV) hypertrophy was observed as a result of the rAAV9-shPLB therapy. Appreciable reduction in cardiac hypertrophy and cardiac fibrosis with normalization of massive cardiac dilation was also observed. Further reduction in both cardiomyocyte size and cardiac collagen was revealed by histological observation. A similar kind of study was carried out by Poller et al. [97], wherein HF was induced by Transaortic banding (TAB) in rats. RNAi was induced by aortic root injection of AdV-shPLB for short term and i.v. injection of AAV9-shPLB for long-term therapy. An increase in survival rate was observed along with reduction in hypertrophy and hemodynamic improvement [97]. In another study, administration of siRNA targeting PLB resulted in the reduction in PLB mRNA level after transfection. Herein, Watanabe et al. [98] transfected NRMCs with siRNA using haemagglutinating virus of Japan (HVJ) envelope vector which is inactive and free of viral genome. A considerable reduction in PLB mRNA (24%) and in PLB protein level (12%) was observed. Further an improvement in uptake rate of SR Ca^{2+} was produced because of increased in affinity of SERCA2 for Ca^{2+}. It was also observed that PLB siRNA in hydrogen peroxide-exposed myocytes induced an increase in SERCA2/PLB ratio with increase in Ca^{2+} uptake affinity. Thus, PLB ablation by siRNA technique may prove to be a novel therapeutic strategy for HF [98]. Andino et al. [99] designed two siRNA candidates—siM248 and siM750—for RNAi against PLB synthesis using the Ambion siRNA design software. Primary neonatal rat ventricular cardiomyocytes (PNRVCs) were isolated from Sprague–Dawley rats and infected with AAV-mediated siRNA. Co-transfection of HEK 293 cells with the PLB expressing plasmid and shRNA plasmids was also carried out. They observed a reduction in PLB amount in both cultures of PNRVCs and murine myocardium on transfection with AAV delivered shRNAS. Further an increase in motion and relaxation rate were recorded because of PLB knockout. Thus, PLB can prove to be a potential target through siRNA technology for treatment of HF.

The IGF system consists of three ligands (Insulin like growth factor-I), IGF-I, IGF-II, and Insulin, and three receptors—IGF-I receptor (IGF-IR), IGF-II/mannose 6-phosphate receptor (IGF-IIR), and insulin receptor. It has been shown that IGF-IIR is involved in apoptosis and tumorigenesis functioning as a death receptor [100]. HF has been found to be accompanied with apoptosis through activation of caspase cascade [101]. It has also been found that disruption of IGF-IIR protein leads to cellular protection against cardiomyocyte apoptosis [102] and hence may be of importance in HF. Chu et al. [103] initially transfected NRVMs with IGF-IR shRNA to prevent the interference of IGF-IR and then specifically activated the IGF-IIR using Leu27 IGF-II analog. The role of IGF-II/IGF-IIR (IGF-II receptor) activation and its downstream signaling was investigated. An increase in cell apoptosis was found to be enhanced by suppression of IGF-IR synergized by IGF-II in NRVMs. RNAi was found to block the Leu27IGF-II-induced cell apoptosis by inhibition of IGF-IIR, α-q polypeptide, or calcineurin. Further siRNA was again developed to inhibit the endogenousGα-q and calcineurin in the NRVMs, which resulted in significant suppression of caspase-3 pathway and apoptosis induced by Leu27IGF-II. Thus, disruption or suppression of IGF-IIR signaling pathway may serve as novel target for preventing progression of HF and myocytes apoptosis.

Nuclear factor (NF)-κB is a transcription factor that has a role in activation of number of genes known to be involved the pathogenesis of cardiac remodeling, and in HF [104]. NF-κB usually is in inactive form in the cytoplasm bound to inhibitory proteins called IκBs (IκBα, IκBβ, and IκBε). These IκB proteins on phosphorylation followed by proteolytic degradation lead to the activation of NF-κB, its translocation into the nucleus, and finally stimulation of expression of various inflammatory genes [105]. Activation of the NF-κB signaling pathway may be associated with the development of cardiac hypertrophy and its transition to HF. Gupta et al. [106] developed Myo-Tg, a transgenic mouse model in whom myotrophin was over expresses, specifically in the heart which initiated hypertrophy in

the myocardium and gradually resulted into HF from chronic hypertrophy.RNAi targeting NF-κB p65 using a lentiviral vector (L-sh-p65) was conducted to explore the effects of inhibition of NF-κB signaling. It was observed that NF-κB p65 gene knockdown resulted in a considerable decrease in heart weight-to-body weight ratio, myocyte growth, and ANF expression. Further an attenuation of the NF-κB activation cascade and improvement in ejection fraction was observed. Thus, NF-κB can prove to be a useful target for treatment for hypertrophy and its progress to HF.

There is a widespread implication of apoptosis regulation by adinopectin in obesity-linked diseases such cardiovascular disease [107]. Also, the loss of cardiomyocytes via apoptosis is a major factor for progressive deterioration of hypertrophied left ventricle, ultimately leading to end-stage cardiomyopathy [108]. Park et al. [109] performed the hypoxia/reoxygenation (H/R) treatment on commercially available H9c2 rat embryonic cardiac myoblasts. Another set of H9c2 cells were pretreated by transfection with plasmid encoding adiponectin receptor (AdipoR1) (C)- Flag and later treatment with globulin adinopectin (gAd) before subjecting them to H/R treatment. Further 21-nucleotide siRNA-sequence design was used to mediate the knockdown of AdipoR1 and APPL1 on the H9c2 cells pretreated with gAd. It was found that adinopectin pretreatment not only prevented H/R-induced apoptosis (assessed by DNA fragmentation and annexin V labeling) but also attenuated H/R-induced cytochrome c release from mitochondria, caspase-3 activity and ROS generation. It was also observed that knockdown of AdipoR1 and APPL1 attenuated the protective effect of gAd, further supporting the fact that gAd has anti-apoptotic actions. Thus, adinopectin via anti-oxidative mechanism exhibits protective effect in H/R-induced apoptosis. Thus, targeting this mechanism may prove beneficial in HF.

MYOCARDITIS

Myocarditis is an inflammatory disorder of the heart muscle and leads to acute HF with chronic dilated cardiomyopathy. Myocarditis initiates as viral disease, and molecular techniques have confirmed viral persistence. Myocyte expresses molecular, cytokine and vascular changes that lead to dilated cardiomyopathy and HF [110].

Coxsackievirus B3 (CVB3), which is a member of the genus *Enterovirus* within the family *Picornaviridae*, is the most common causal agent of viral myocarditis, but existing drug therapies are of limited value. Differences in host susceptibility to viral myocarditis are caused by a given strain of CVB3 and are known to be largely related to host genetic factors [111]. Yuan et al. [112] investigated whether RNAi can protect against CVB3 infection by selecting various sequences at different locations in CVB3 genome that may have important functions in viral replication. RNAi effect on viral replication was evaluated in HeLa cells and murine cardiomyocytes by using total of five siRNAs targeting 5_UTR, start codon, viral capsid protein VP1, viral protease 2A, and RdRP regions. The antiviral activity of siRNAs which inhibited the replication of virus in HeLa cells was indicated by the observation of decreased virus titer for all CVB3-specific siRNAs except siRNA-2 when compared with the supernatant which was treated with control siRNAs or left untreated. Further the potency of siRNAs was determined by transfecting HeLa cells with siRNA-4 succeeded by CVB3 infection. An increase in amount of siRNA-4 as observed with subsequent decrease in virus titer in supernatant and viral protein (VP1) protein in cells which suggested the siRNAs potency. They also evaluated the ability of siRNAs to inhibit viral replication post viral infection by transfecting CVB3 infected HeLa cells with siRNA-4 or siRNA-C. Reduction in VP1 expression and virus titer indicated the siRNAs ability to inhibit progressive viral infection. CVB3 being a cardiotropic virus and commonly inducing viral myocarditis, Yuan et al. [112] also evaluated these siRNAs in the cardiomyocyte cell line of HL-1 mouse by initial transfection with siRNA followed by CVB3 infection and it was found that the pattern for inhibition resembled to that observed in HeLa cells. Thus, siRNA technology can serve as a good therapeutic potential for CVB3 infection by inhibiting its proliferation in myocarditis.

Schubert et al. [113] evaluated the knockdown efficiencies of two chemically synthesized siRNAs, siRNA2, and siRNA4 against regions on the viral mRNA that codes for the 3D-RdRP of CVB3. A reduction of 90% and 80% in virus titer was observed by transfection of virus infected HeLa cells individually with siRNA2 and siRNA4, respectively. Further they evaluated the effect of siRNA inhibition on RdRP kockdown by constructing a siRNA double expression vector (SiDEx) that expressed both the siRNAs from a single plasmid. It was observed that although the knockdown efficiency by SiDEx was comparable to the inhibition of individual

Table IV Summary of the siRNA target genes for hypertrophy, heart failure and myocarditis.

Disease	Target	Model / Process	Effect	Reference
Hypertrophy	PDE1A	Neonatal and adult rat ventricular myocytes	Prevented hypertrophy	[85]
	TACE	Rodent models of spontaneous and agonist-induced hypertension	Stopped development of hypertrophy, inhibited expression of ADAM-12 and MMP-2	[86]
	L-type calcium channel accessory β subunit	PPT.CG.H1.β_2 – injected rats	Attenuate hypertrophy, ↓ left ventricular wall thickness & heart wt/body ratio	[87]
	Atrogin-1	Transgenic mice	↓calcineurin protein level and blunted cardiac hypertrophy	[89]
	MCIP1 gene	Cultured rat cardiomyocytes	Reversed E2 restraint of protein synthesis and inhibition of Ang II-induced calcineurin activity	[90]
	E2F1, cyclin E1, cycin E2 genes	Synchronous & asynchronous CSMCs	↓ S phase, ↑G_1-G_0 phase	[91]
	PAI-1	PASMCs in idiopathic PH	↓ PASMC migration, ↑ PASMC proliferation	[92]
Heart Failure	PLB	Primary neonatal rat ventricular cardiomyocytes	↓PLB protein, ↑cell motion and relaxation rates	[99]
	PLB	Rat model of transaortic banding	↓PLB protein, suppression of sarcoplasmic reticulum Ca^{+2} ATPase, ↓ cardiac hypertrophy, cardiomyocyte diameter & cardiac fibrosis	[96]
	PLB	Rat model of transaortic banding	Hemodynamic improvement, ↓ hypertrophy	[97]
	PLB	Neonatal rat myocytes	↓ PLB mRNA level, ↑ Ca^{+2} uptake affinity, ↓ SERCA2 protein level	[98]
	IGF-receptor	Neonatal rat ventricular myocytes	Inhibits IGF-II receptor, IGF-I receptor, blocks Leu 27IGF-II induced cell apoptosis	[103]
	NF-κB p65	Transgenic Myo-Tg	Regression of cardiac hypertrophy	[106]
	AdipoR1 and APPL1	H9c2 rat embryonic cardiac myoblasts	↓ protective effect of gAd	[108]
Myocarditis	3D-RNA dependent RNA polymerase	HeLa cells	Inhibition of virus propagation, ↓ virus titre by 90%	[113]
	Viral genome, viral protease 2A	HeLa cells & murine cardiomyocytes	92% inhibition of CVB3 replication	[112]

PDE1A, phosphodiesterase 1A; TACE, tumor necrosis factor-α-converting enzyme; ADAM-12, a disintegrin and metalloproteinases-12; MMP-2, matrix metalloproteinases-2, Ang II, angiotensin II; PLB, phospholamban; ATPase, adenosine triphosphatase; mRNA, micro-ribonucleic acid; Ca^{+2}, calcium; SERCA2, sarcoplasmic reticulum calcium-transporting ATPases; IGF-receptor, insulin like growth factor receptor; NF-κB p65, nuclear factor κB p65; CVB3, coxsackievirus B3; ↓, decrease; ↑, increase.

mono-expression vectors, it was not significantly strong and less effective at lower concentrations of vector. But SiDEx was found to be effective against artificially induced mutation in target site where single shRNA proved to be ineffective. Thus, they concluded that sustained silencing of virus and viable viral mutants could be achieved in myocarditis by employing SiDEx. *Table IV* summarizes application and target genes of siRNA for hypertrophy, HF and myocarditis.

LIMITATIONS OF siRNA

Foremost hurdle in siRNA therapy is the issue of siRNA delivery as far as its therapeutic use is concerned. Having anionic and hydrophilic nature, siRNA fails to enter cell by direct passive diffusion. Further reduced penetration across the capillary endothelium, inefficient uptake by tissue cells, rapid enzymatic degradation in plasma and enhanced renal elimination are problematic issues for delivery of naked siRNA in vivo [114]. Thermodynamic stability, half-life, knockdown, tissue-specific delivery, consistent expression, toxicity, and high cost factor are some of the other related issues in it delivery obstacles.

siRNA designing poses a greater challenge for researchers at various levels ranging from identification of proper gene target, designing specific siRNA of required sequence, mismatch between the target mRNA and siRNA, selection of siRNA length, off-target effects and innate immunity, etc. It has been observed that siRNAs can induce innate immune pathways by activation of alternative dsRNA cellular pathways.

This is a result of up-regulation of large number of other genes other than the target gene [115]. siRNA off-target relates to inhibition of any target that is affected by siRNA other than the assigned target because of partial homology, which this gene shares with siRNA [116]. In such cases, genes other than target gene show altered expression. Complete and partial off-target mechanisms have been reported to exhibit such phenomenon.

CONCLUSIONS

Cardiovascular diseases like hypertension, stroke, HF, myocarditis, etc., have been on rise since decades and have taken a heavy toll on human lives, thereby reducing the life span. Modern science has been successful in prevention and treatment for these deadly diseases through various target-specific drugs, but till today, no conditions of complete recovery or prevention of relapse has been achieved. Moreover, they are accompanied with the risk of side effects. Surgical interventions to some extent have proved beneficial, but they are also associated with danger to the life of patient.

Given its ability to knockdown any gene of interest, siRNA can be used to target IL-6, IL-13, Pim-1, MEK, MMP-7, etc., to cure HT from its key inducing factors and attenuate its acceleration. Further silencing of some newer targets by RNAi including ESELE, ICAM-1, VCAM-1, CRP, Omi-HtrA2, CECs, etc., can also be explored in depth to eliminate the harmful effects of atherosclerosis and thereby reduce the risk of other accompanying disorders. siRNA can also be explored to a larger extent for targeting TG2, caspase-3, MMP-9, CXCR4, HMGB1, etc., and thus ablate the deleterious effects of stroke, thus improving the survival rate. Knockdown of PDE1A, TACE, E2F1–Cyclin E1–Cyclin E2, etc., by RNAi in hypertrophy can help gain advantage over the existing drug therapies by altering the disease from root cause. siRNA targeting of PLB, NF-κB, IGF-IIR pathway in HF, and CYB3 genome in myocarditis can prove to be effective in improving diseased condition and decrease mortality rate. The development of siRNA and its delivery techniques thus may serve as promising targets in future. This strategy may be effectively used to identify drug targets or new components of signaling pathways. Although in infancy stage, the scientific community considers RNAi, a breakthrough biological discovery of the decade with the potential to change how diseases are treated. With the advancements in the approaches designed for the delivery of siRNAs, these could serve as potential drugs for the treatment for CVDs and thus reduce the burden of such diseases globally.

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