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Targeting and delivery of microRNA-targeting antisense oligonucleotides in cardiovascular diseases

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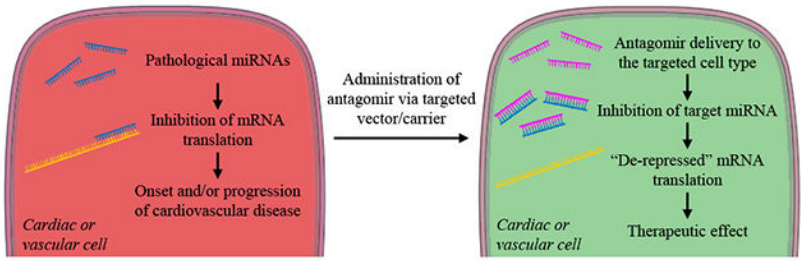
Abstract

Discovered three decades ago, microRNAs (miRNAs) are now recognized as key players in the pathophysiology of multiple human diseases, including those affecting the cardiovascular system. As such, miRNAs have emerged as promising therapeutic targets for preventing the onset and/or progression of several cardiovascular diseases. Anti-miRNA antisense oligonucleotides or “antagomirs” precisely block the activity of specific miRNAs and are therefore a promising therapeutic strategy to repress pathological miRNAs. In this review, we describe advancements in antisense oligonucleotide chemistry that have significantly improved efficacy and safety. Moreover, we summarize recent approaches for the targeted delivery of antagomirs to cardiovascular tissues, highlighting major advantages as well as limitations of viral (i.e., adenovirus, adeno-associated virus, and lentivirus) and non-viral (i.e., liposomes, extracellular vesicles, and polymer nanoparticles) delivery systems. We discuss recent preclinical studies that use targeted antagomir delivery systems to treat three major cardiovascular diseases (atherosclerosis, myocardial infarction, and cardiac hypertrophy, including hypertrophy caused by hypertension), highlighting therapeutic results and discussing challenges that limit clinical applicability.

Graphical Abstract



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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Keywords

microRNA; antagomir; targeted delivery; encapsulation; cardiovascular diseases

1. Micro-RNAs (miRNAs) as therapeutic targets

1.1 Biogenesis and activity of miRNAs

Only 1-2% of the mammalian genome encodes proteins, whereas the remainder is untranslated. These untranslated genomic regions contain numerous sequences that are transcribed into “noncoding RNAs” (ncRNAs). NcRNAs are classified based on length into small RNAs [18-200 bp; including “microRNAs” or “miRNAs”, piRNAs, snoRNAs, tRNA-derived fragments (tRFs), and likely others] and long RNAs (>200 bp; “long non-coding RNAs” or “lncRNAs”). MiRNAs are primarily involved in the posttranscriptional regulation of protein-coding genes (1). Consistent with their roles in regulating critical biological processes, expression of individual miRNAs is tightly regulated in tissue- and developmental stage-specific patterns. For example, several miRNAs (e.g., miR-1, miR-133, and miR-499) are expressed during embryonic heart development, in which they regulate fundamental processes of cardiogenesis such as cardiomyocyte proliferation and differentiation (2). As described in greater detail elsewhere (1), miRNA-encoding sequences are transcribed within the nucleus by RNA polymerase II to generate primary miRNAs (pri-miRNAs) that are first cleaved by Drosha, a class 2 ribonuclease III enzyme, to become precursor miRNAs (pre-miRNAs). Pre-miRNAs are exported to the cytosol by exportin 5, where they are further cleaved by Dicer (also part of the ribonuclease III family) into double-stranded mature miRNAs. One of the miRNA strands is then loaded onto the RNA-induced silencing complex (RISC), guiding RISC to a target messenger RNA (mRNA). The “seed” sequence of the miRNA, located between nucleotides 2 and 7, binds to a complementary sequence in the 3’-untranslated region of a target mRNA, inhibiting mRNA translation (1). Importantly, a single miRNA can target multiple mRNAs, simultaneously altering levels of several proteins in single or multiple biological pathways. For example, by repressing SOX6, miR-208 activates cellular signaling that stimulates proliferation while simultaneously inhibiting pro-apoptotic pathways by repressing CDKN1A (3). MiR-223 also has a multifaceted role, regulating cholesterol metabolism via targeting SCARB1 (repressing high-density lipoprotein uptake) and by targeting HMGCR (lowering cholesterol biosynthesis) (4). In the vascular wall, miR-155 modulates distinct biological processes that shape the endothelial phenotype. Through direct inhibition of NOS3, miR-155 represses vasorelaxation (5), whereas it induces endothelial inflammation by targeting the transcription factor Bcl6 (6).

1.2 Roles of miRNAs in cardiovascular homeostasis and disease

MiRNAs regulate numerous molecular pathways that help maintain cardiovascular homeostasis. Accordingly, dysregulation of miRNAs can significantly alter cellular physiology, triggering the onset and progression of cardiovascular disease (CVD). Because the pathophysiological relevance of miRNAs in CVDs is discussed extensively in previous reports (7–11) and to limit the scope of this review, herein we focus primarily on the role of miRNAs in three major CVDs: atherosclerosis, myocardial infarction (MI) and

its complications (including arrhythmias and fibrosis), and cardiac hypertrophy, including hypertrophy caused by hypertension. A focus on these 3 CVDs also allows us to highlight major issues and approaches in targeting and delivery of antagomirs to cardiac and vascular cells. Because of space limitations, here we focus on a subset of miRNAs that are relevant to these 3 CVDs, selecting miRNAs that are clearly connected both with mRNA targets and with regulation of cardiovascular physiology and disease.

MiRNAs regulate several biological processes that contribute to atherosclerosis (Table 1) (11); some of these processes are specific to endothelial cells (EC). For example, miR-10a maintains EC homeostasis and helps prevent atherosclerosis by downregulating the NFKB1 activators MAP3K7 and BTRC, thereby reducing levels of EC adhesion molecules [e.g., E-selectin and vascular cell adhesion molecule-1 (VCAM1)]. MiR-10a also reduces expression of proinflammatory cytokines (e.g., IL6 and CCL2) (12). Supporting an atheroprotective role for miR-10a, humans with coronary artery disease (13) have lower levels of miR-10a both in serum and coronary artery endothelium (14). MiR-126 also has atheroprotective activity, reducing atherosclerosis in mice by blocking the Notch1 inhibitor Dlk1 in EC, leading to increased EC proliferation that may improve vascular healing (15). Consistent with this mechanism, miR-126 expression in human carotid artery lesions correlates inversely with DLK1 positive EC (15).

MiRNAs also regulate atherogenesis via their activities in macrophages, including effects on lipid metabolism (16). For example, miR-155 (which is upregulated by Ox-LDL) accelerates atherosclerosis by targeting Hbp1, leading to enhanced lipid uptake in murine macrophages, increased generation of reactive oxygen species, and enhanced foam cell formation (17). Circulating monocytes of CAD patients have elevated levels of miR-155, supporting an atherogenic role for miR-155 in humans (17). Similarly, miR-33a contributes to atherogenesis by reducing macrophage ABCA1 expression, lowering cholesterol efflux, and sustaining foam cell formation (18).

The contribution of vascular smooth muscle cells (VSMCs) to atherogenesis depends on their phenotype, which is tightly regulated by miRNAs. For example, miR-145 maintains the differentiated state of VSMCs via inhibition of the transcription factor KLF5, preventing neointima formation in balloon-injured rat carotid arteries (19). MiR-145 also has biological roles outside the cardiovascular system. For example, inhibition of KLF5 by miR-145 represses proliferation and migration of cervical and nasopharyngeal carcinoma cells (20, 21). MiR-362 also plays an atheroprotective role by inhibiting ADAMTS1, thereby repressing VSMC proliferation and migration into the intima (22). MiR-362 levels are decreased in plasma of CAD patients, consistent with an atheroprotective role (22).

MiRNAs play significant roles in the regulation of cardiac biology and disease, including ischemic heart disease and MI (Table 1) (23). For example, muscle-enriched miR-1 impairs cardiac contractility by repressing Kcnj2 and Gja1 proteins that form K^+ and gap junction channels, respectively, slowing cardiac conduction and promoting arrhythmias after MI in rats (24). Moreover, miR-1 is upregulated in the myocardium of patients with CAD and its levels are inversely correlated with levels of KCNJ2 and GJA1 proteins, suggesting a detrimental role for miR-1 in humans with ischemic heart disease (24). Cardiac remodeling

after MI is altered by miRNAs such as miR-433, which—at least in part by inhibiting Azin1—increases Tgf-ß1, leading to Smad3 activation and cardiac fibrosis after experimental MI in mice (25). Interestingly, miR-433 is upregulated both in the plasma of patients with CAD and in the left ventricle of patients with nonischemic dilated cardiomyopathy, suggesting that miR-433 might contribute to both ischemic and non-ischemic left ventricular dysfunction (13, 25). MiRNAs also regulate the myocardial angiogenic response to ischemia. For example, miR-24 is upregulated in ECs of ischemic mouse myocardium, and impairs neovascularization after experimental MI, in part by promoting EC apoptosis. These effects are probably mediated via direct inhibition of the transcription factors Gata2 and Pak4, which leads to decreased expression of Hmox1 (antioxidant enzyme) and activation of Bad (a pro-apoptotic protein), respectively (26). MiR-134 also inhibits myocardial angiogenesis after experimental MI in mice, likely by repressing Kdm2a, leading to reduced expression of vascular endothelial growth factor A (27). Importantly, miR-134 also exerts an anti-angiogenic role in non-cardiac pathologies such as osteosarcoma (28), raising the possibility that efforts to improve myocardial vascularization by targeting miR-134 could have harmful off-target effects.

MiRNAs also contribute to the regulation of blood pressure and—by extension—to the pathogenesis of hypertension and hypertensive heart disease (Table 1). MiRNAs help regulate the renin-angiotensin-aldosterone system, which plays a central role in the pathogenesis of essential hypertension. Marques et al. identified miR-181a and miR-663 as repressors of renin and found decreased expression of both miRNAs in the renal cortex of hypertensive versus normotensive patients (29). MiR-143 directly targets angiotensin converting enzyme 2, and miR-143 levels are lower in plasma of patients with essential hypertension (30). MiRNAs also regulate pathways that contribute to hypertension-induced cardiac hypertrophy. For example, miR-26 inhibits the transcription factor Gata4 (a well-established promoter of cardiomyocyte growth) and prevents cardiac hypertrophy in mice subjected to cardiac pressure overload (31). In turn, miR-217 promotes cardiac hypertrophy by targeting Pten, thereby activating the AKT signaling pathway (32), and mir-214 enhances angiotensin II-induced cardiac hypertrophy in mice by targeting Sirt3, leading to mitochondrial dysfunction (33).

In summary, miRNAs are key regulators of cardiovascular pathophysiology, acting by downregulating components of critical pathways that either promote or prevent several CVDs, including atherosclerosis, MI and its sequelae, and hypertension and its sequelae. Accordingly, miRNAs are attractive targets for development of novel cardiovascular therapies. However, their roles in other pathologies such as cancer must be considered as miRNA inhibitory strategies are translated into human therapies.

2. MiRNA-targeting therapeutics: structure, mechanism, chemical modifications, and toxicity

2.1 Agomirs and antagomirs: structure and mechanism of action

Therapies that target miRNAs aim either to increase the expression/abundance of miRNAs that prevent disease or to reduce the activity of miRNAs that cause disease. These

therapies employ miRNA mimics or miRNA inhibitors, respectively. MiRNA mimics (or agomiRs) are synthetic double-stranded small RNA molecules that specifically match the sequence of endogenous mature miRNAs. MiRNA inhibitors are classified as either antisense oligonucleotides (ASOs) or miRNA sponges based on their molecular structure (34). MiRNA-targeting ASOs or antagomirs are single-stranded small RNAs that bind complementarily to their miRNA targets, blocking interaction of these targets with their corresponding endogenous mRNA targets, thereby increasing (or "de-repressing") expression of these mRNA targets. Similarly, miRNA sponges possess multiple miRNA binding sequences that can sequester multiple copies of either a single miRNA species or several different miRNA species. Accordingly, sponges are potentially a more potent approach for reducing levels of target miRNA. Although miRNA mimics (i.e., agomiRs) have shown potential in preclinical studies and are beginning to be tested clinically (35, 36), they have not yet been tested in clinical trials for treatment of CVDs. For this reason, and to limit the scope of this review, we will focus on miRNA inhibitors rather than agomiRs. Among miRNA inhibitors, we will discuss antagomirs rather than sponges because antagomirs are far more widely used both in preclinical studies and clinical trials. Moreover, because many advances in antagomir-based therapies were originally developed for mRNA-targeting ASOs, some of the studies cited herein involve mRNA-targeting ASOs.

2.2 Chemical modification of ASO antagomirs

Unmodified RNA molecules injected *in vivo* are quickly degraded by endogenous nucleases (37). Accordingly, unmodified ASO antagomirs administered via intravenous or subcutaneous injection have limited bioavailability and are unlikely to reach their miRNA targets. Moreover, ASOs have an overall negative charge that hampers cellular uptake, requiring injection of high doses to achieve biologically meaningful effects. To overcome these challenges, several chemical modifications are used to improve ASO stability, increase cellular uptake, and enhance affinity for target miRNAs. These modifications (38) are applied to the backbone as well as the ribose and/or nucleobase of ASOs and include: (1) changes in the phosphodiester bonds such as incorporating a phosphorothioate internucleotide linkage to confer resistance to nuclease degradation and increase bioavailability; (2) modification of ribose to enhance ASO stability and affinity for target miRNA (e.g., 2´-O-methyl, 2´-O-methoxyethyl, and 2´-fluoro modifications); (3) construction of a locked nucleic acid (35) by placing a methyl bridge between the 2´-O and 4´ position of the ribose ring, thereby increasing the affinity of LNAs for their target miRNAs; and (4) substitution of the ribose-phosphate backbone with a pseudopeptide sequence [creating a peptide nucleic acid (PNA) molecule], or with a 6-membered morpholino ring and phosphorodiamidate linkages, generating a phosphorodiamidate morpholino oligomer (PMO). Both PNAs and PMOs are neutrally charged and are resistant to cleavage by nucleases (39). All the aforementioned chemical modifications of ASOs have been thoroughly described in previous reviews (38, 40).

2.3 Antagomir toxicity

Despite the above advancements in ASO chemistry, toxicity remains a major barrier to clinical application. Because ASOs accumulate primarily in liver and kidney after intravenous or subcutaneous injection, they may have toxic effects in these organs, which

may be exacerbated by high doses (41). LNA-modified ASOs may induce hepatotoxicity, potentially by increasing hepatic expression of apoptotic proteins (e.g., caspase 3), although the underlying molecular mechanisms that drive hepatotoxicity remain unclear (42). Moreover, circulating ASOs also alter platelet activity, causing thrombocytopenia in humans (43). *In vitro* studies suggest that thrombocytopenia is a consequence of platelet hyperactivation and aggregation that are stimulated via ASO activation of TLR-7, -8 and -9 (44). The toxicities of ASOs also include off-target effects caused by hybridization with RNA molecules other than their intended targets (45). For all of these reasons, extensive preclinical assessment of the toxicity of an ASO antagomir is required before it can be administered to humans.

To diminish their toxicity while simultaneously enhancing their biological activity, ASOs can be chemically linked to biomolecules that target the ASOs to specific tissues or cell types. For example, ASOs can be targeted via conjugation to aptamers, single-stranded oligonucleotides (20-100 nt) that bind specific target proteins with high affinity. Accordingly, aptamers enhance delivery of ASO to cells that express a specific protein on their surfaces (46). Similarly, conjugation of ASOs to N-acetylgalactosamine, a ligand of the liver-enriched asialoglycoprotein receptor (ASGR1), enhances ASO delivery to human hepatocytes (47, 48). Lucas et al. used a different approach to achieve site-specific ASO activity: construction of a light-activatable ASO by attachment of photolabile protecting groups or “cages” to the ASO nucleobases (Fig. 1A) (49). These cages prevent duplex formation with the target miRNA but can be removed by irradiation with 325 nm light. Intradermal injection of a caged anti-miR-92a antagomir in diabetic mice significantly reduced miR-92a levels in irradiated skin and improved wound healing similarly to conventional anti-miR-92a. However, mice injected with the light-activated anti-miR-92a had unchanged miR-92a expression in liver and kidney whereas mice injected with conventional anti-miR-92a had significantly lower expression of kidney miR-92a. The authors speculated that this approach could be used—likely with the aid of devices such as catheters or endoscopes—to achieve site-specific activity of antagomirs in internal organs while avoiding toxicity caused by systemic distribution of antagomirs.

Modifications to ASOs that increase cellular uptake and bioavailability could also reduce toxicity by reducing their circulating half-life and systemic distribution and by allowing administration of lower doses. Conjugation of ASOs to cholesterol improves their cellular uptake and endosomal escape, leading to increased intracellular availability in several cell types including cardiomyocytes (50). Intraperitoneal administration of cholesterol-modified anti-miR-199b ASO effectively reduced miR-199b levels in mouse hearts, outperforming unconjugated ASOs (51). Betts et al. attached an arginine-rich cell-penetrating peptide to uncharged ASOs, enhancing their delivery to murine hearts after intravenous injection (52). These are promising results; however, both cholesterol conjugation and attachment of a cell-penetrating peptide would likely enhance antagomir entry into both targeted and non-targeted cells, potentially increasing off-target effects and toxicity.

The above-described advancements in the chemistry and targeting of ASOs have, in some cases, allowed ASOs to advance to clinical application (53, 54). However, clinical use of

ASOs to treat CVDs remains limited, and use of miRNA-targeting ASOs (i.e., antagomirs) continues to be challenged by toxic effects and limited efficacy (55).

3. ASO delivery systems

3.1 ASO delivery to cardiac or vascular cells

Intravenous or subcutaneous administration of non-encapsulated ASOs/antagomirs remains the most common delivery method in preclinical experimental models and clinical studies. The most clinically successful ASO delivery approach to date uses subcutaneous injection of ASOs that are targeted to the liver by conjugation to N-acetylgalactosamine. These ASOs are taken up by the hepatocyte-expressed asialoglycoprotein receptor ASGR1, as described above. Targeted ASO/antagomir delivery to cardiac or vascular cells has proved more challenging because neither cardiac nor vascular cells express cell-type-specific receptors that internalize ligands similarly to ASGR1. Lack of cardiovascular equivalents to ASGR1 has prompted development of alternative delivery systems in which ASOs/antagomirs are either expressed by a viral vector that has tropism for specific cardiac or vascular cell types or are encapsulated in non-viral nanoparticles (NPs) that are constructed with a goal of targeting their ASO/antagomir cargo to one of these cell types (56). The viral vectors contain and express nucleotide sequences encoding the ASOs/antagomirs, whereas the NPs can carry either the ASOs/antagomirs themselves (as synthetic oligonucleotides) or can deliver plasmid constructs encoding ASOs/antagomirs, allowing longer-term expression as well as cell type-specific expression by using promoters that are exclusively expressed in a desired cell type. Use of either viral vectors or NPs to deliver ASO/antagomirs prevents their destruction by endogenous nucleases, facilitates cellular uptake, and reduces endosomal/lysosomal degradation. Therefore, both viral vectors and NPs can increase cell-type-specific delivery to cardiac and vascular cells while enhancing intracellular bioavailability. Specific viral vectors and NPs have both advantages and limitations that must be considered when designing a strategy for delivering ASOs to cardiac and vascular cells (Table 2). In addition, both viral vectors and NPs can be delivered via multiple routes including parenteral injection with systemic distribution, local injection in proximity to the target cells, and device-assisted local delivery. In this section, we discuss viral and non-viral delivery systems as well as strategies for delivery of ASOs/antagomirs to cardiac and vascular cells (Fig. 1).

3.2 Viral vectors

Adenoviruses (Ads) and adeno-associated viruses (AAVs) are the most commonly used viral vectors for delivering genes to cardiovascular cells (Fig. 1B). Both vectors can transduce quiescent cells such as ECs, VSMCs, or cardiomyocytes (57). Ads are non-integrating double-stranded DNA vectors with high vascular-cell and cardiomyocyte transduction efficiency. First-generation Ads contain most of the native viral genome, have limited capacity for transgene insertion (~ 7 kb), achieve only short-term transgene expression (1 to 2 weeks), and induce a strong inflammatory response in the vascular wall (58). In contrast, 3rd generation or helper-dependent Ads lack all viral protein-coding sequences, have a large capacity for transgene insertion (up to ~35 kb) (59), and achieve long-term (at least 48 weeks) transgene expression in preclinical models of vascular wall gene transfer, with a minimal local inflammatory response (60). Serotype 5 Ads are typically used to deliver

genes to cardiac and vascular cells. Importantly, effective transduction by serotype 5 relies on the presence of the Coxsackievirus and adenovirus receptor (CAR) on the surface of targeted cells. CAR expression is high in cardiomyocytes, but is also present at adequate levels in ECs and VSMCs (61). Other Ad serotypes, such as type 35, show higher tropism towards VSMCs, through interaction with the CD46 receptor, and may therefore be more suitable for transducing the arterial intima and media (62). More-precise targeting of Ads to ECs and VSMCs (and more-efficient transduction) may be achieved by modification of capsid proteins to target molecules that are abundant on the surfaces of these cell types, including heparan sulfate and angiotensin-converting enzyme (63, 64). As an example of successful Ad-mediated antagomir delivery into cardiomyocytes, intramyocardial injection of Ads encoding anti-miR-327 and anti-miR-98 effectively depressed expression of their corresponding target miRNAs *in vivo* (65, 66).

AAVs are single-stranded DNA vectors that can achieve long-term transgene expression. AAVs have lower immunogenicity than Ads, although their capacity for transgene insertion is limited to ~4.7 kb (59). However, for expression of an antagomir, 4.7 kb of packaging capacity is more than sufficient. Several AAV serotypes display high cardiac tropism, especially AAV1, AAV6, AAV8 and AAV9. Cardiomyocyte transduction by these AAVs is facilitated by binding of their capsids to proteins on the cardiomyocyte surface including the laminin receptor or N-linked sialic acid (67). The ability of wild-type AAVs to transduce vascular cells appears relatively low in preclinical models, although some serotypes such as AAV1 and AAV5 are reported to transduce rabbit vascular media and adventitia *in vivo* (68). Modifications of the AAV2 and AAV9 capsids have improved their tropism towards ECs (69–71). For example, Krolak et al. modified the AAV9 capsid, enhancing its capacity to transduce cultured human microvascular ECs by over 70-fold (71). Moreover, intravenous injection of the modified AAV9 yielded high *in vivo* transduction of arteries, veins and capillaries in murine brain and of aortic endothelium (71). These modified AAVs have great potential for improving the delivery of antagomirs to cardiac and vascular cells. Examples of the use of AAVs to deliver ASOs to cardiomyocytes include repression of miR-20b in murine myocardium after intravenous injection of an AAV encoding anti-miR-20b (72) and injection of an AAV9 encoding anti-let-7i-5p into murine myocardium, which de-repressed cardiac expression of the let-7i-5p target genes E2f2 and Ccnd2 (73).

Lentiviral vectors (LVs) are enveloped single-stranded RNA viruses with high packaging capacity (~10 kb) that achieve long-term transgene expression in both quiescent and proliferating cells, in part due to integration of the LV genome into the host cell genome (59). However, genomic integration of LVs can potentially lead to insertional oncogenesis (74). This limitation of LVs has been addressed by generation of non-integrating LVs. Non-integrating LVs achieve long-term transgene expression in non-dividing cells and—to a lesser extent—in dividing cells (74, 75). Moreover, LVs have relatively low immunogenicity, low prevalence of pre-existing neutralizing antibodies, and can be easily pseudotyped (i.e., their envelopes can be modified to include surface proteins from other viruses such as the vesicular stomatitis virus surface glycoprotein), altering their tropism and reducing recognition by the immune system (75). LVs can deliver antagomirs to ECs and VSMCs *in vitro* (76, 77). However, *in vivo* transduction efficiency of cardiac and vascular cells by LVs appears to be limited (78, 79). Intravenously injected LVs primarily transduce liver,

spleen, and bone marrow (80). Compared to Ads and the cardiotropic AAVs (e.g., AAV2 and AAV9), LVs inefficiently transduced murine cardiomyocytes after intramyocardial injection (79). Whether intravenously injected LVs can transduce cells in the aorta is controversial (78, 81). Redondo et al. reported highly efficient LV-mediated transgene expression throughout the murine aortic wall after intravenous LV injection (81); however, presence of the LV genome in the aorta was never documented. In contrast, we found that LV genomes and LV-mediated transgene expression were both undetectable in murine aortas 4 days after intravenous injection of high-titer LVs—including LVs supplied by Dr. Redondo—casting doubt on whether any cells in the aorta are transduced by intravenously injected LVs (78). Pseudotyping is a potential strategy for increasing the *in vivo* transduction efficiency of LVs. For example, local perfusion of a LV pseudotyped with the bunyaviral Hantaan virus glycoprotein efficiently transduced rabbit carotids after balloon injury (82).

In summary, viral vectors can be used to express therapeutic antagomirs within specific cardiac and vascular cells. Use of antagomir-expressing viral vectors eliminates the need for resource-intensive synthesis and purification of clinical-grade antagomirs. Moreover, a single viral vector injection could achieve long-term *in vivo* expression of an antagomir, leading to durable suppression of its miRNA target. A disadvantage of viral vector delivery systems is that it is challenging to turn them “off” if antagomir expression either becomes unnecessary or is toxic. Moreover, clinical application of viral vector-mediated gene therapy is still hampered by intense systemic innate and adaptive immune responses to viral vector proteins and by the existence of high levels of neutralizing antibodies to Ad and AAV vectors in many humans. These challenges highlight the need for development of non-viral vectors for delivering antagomirs to cardiac and vascular cells.

3.3 Liposomes

Liposomes are synthetic lipid bilayer particles that can encapsulate a variety of molecular cargoes, including antagomirs (Fig. 1C) (83). Because of their high biocompatibility, loading capacity, and relative ease of preparation, liposomes are the most widely used non-viral ASO delivery system. Liposomes are typically synthesized from cationic lipids that entrap ASOs by electrostatic force. Liposomes are often coated with polyethylene glycol (PEG), which confers resistance to degradation by the mononuclear phagocytic system and enhances circulation lifetime (83). The positive charge of liposomal lipids facilitates binding to the negatively charged cellular plasma membrane. Liposomes can also be targeted to specific cell types by covalent conjugation to antibodies or to peptides that bind specific cell-surface antigens. For example, intravenously injected PEGylated liposomes covalently attached to an angiotensin II type 1 receptor binding peptide specifically targeted infarcted myocardium in mice, whereas healthy hearts were not targeted by these liposomes (84). Ko et al. extended this method by conjugating liposomes to both an anti-cardiac myosin antibody and a cell-penetrating peptide. These bivalent liposomes had enhanced uptake in rat ischemic cardiomyocytes after intravenous injection (85). Conjugation of liposomes to antibodies or ligands can also enhance liposome delivery to vascular wall cells including macrophages and ECs (86–88). For example, incorporation of an oxidized phospholipid (a ligand for the CD36 receptor) into liposome-like nanoparticles increased their accumulation in atherosclerotic mouse aortas, presumably by facilitating uptake by intimal macrophages

(87). Liposomes conjugated with ligands of the transferrin receptor (enriched in brain endothelium) cross the blood brain barrier via transferrin receptor-mediated endocytosis, facilitating ASO delivery to the brain (89).

Despite their overall biocompatibility, cationic liposomes can have both cytotoxic and immunogenic effects. These effects are caused by inclusion of synthetic components in liposomes and by their positive charge. These factors drive liposome accumulation in the liver and spleen, promoting complement activation and rapid clearance by the phagocytic system (90). These issues can be mitigated by use of liposomes composed of neutral or ionizable lipids (i.e., lipids that are positively charged at acidic pH—facilitating the incorporation of ASOs into liposomes—but are neutrally charged at physiological pH). For example, Fisher et al. generated neutral lipid NPs conjugated with a VSMC-targeting peptide. These NPs had less *in vitro* cytotoxicity compared to cationic liposomes (91).

3.4 Extracellular vesicles

Extracellular vesicles (EVs) are released from all cell types and resemble liposomes in that they consist of a lipid bilayer surrounding a molecular cargo (Fig. 1C). EVs are derived from either the plasma membrane or from intracellular multivesicular bodies; the latter are termed exosomes (92). Endogenous EVs contribute to intercellular communication and because they are cell-derived products EVs are highly biocompatible. As with liposomes, EVs can be used *in vivo* as vehicles for delivering ASOs to target cells. EVs can be easily isolated from multiple body fluids (e.g., blood, urine or cerebrospinal fluid) using methods such as ultracentrifugation and/or size-exclusion chromatography (92). Alternatively, EVs can be isolated from medium conditioned by cultured cells. This is an attractive approach for obtaining large amounts of high purity EVs because it avoids contamination of EV preparations by apolipoproteins or Tamm-Horsfall protein which are commonly co-isolated with EVs from blood and urine, respectively (93).

Incorporation of antagomirs into EVs can be achieved by i) direct transfection or ii) transfection of cultured cells with an antagomir-encoding plasmid followed by collection of cell-conditioned medium and purification of EVs. The yield of ASO-containing EVs can be increased by inclusion in the ASO sequence of a tetranucleotide (termed the X-motif) that favors incorporation of oligonucleotides into EVs (94, 95). As with liposomes, EVs can be modified to target specific cell types via incorporation of surface-exposed ligands (40). For example, Kim et al. generated small EVs that were coated with a cardiac-targeting peptide and showed that incorporation of the peptide enhanced delivery of intravenously injected EVs to murine hearts (96). Although EVs are promising vehicles for delivery of ASOs *in vivo*, their use as biopharmaceuticals is limited by the difficulty of generating biochemically similar batches of EVs (97). Heterogeneity of EV preparations complicates their use in preclinical studies and is a major regulatory barrier to their use in humans (97).

3.5 Polymer nanoparticles

Polymer nanoparticles (pNPs) differ from liposomes and EVs by not encapsulating ASOs in lipid bilayers, but instead carrying ASOs as cargo attached to a polymeric scaffold (Fig. 1C). PNPs composed of poly(lactic-co-glycolic acid) (PLGA) (a polymer approved

for human use by both the FDA and EMA) have emerged as attractive alternatives to liposomes and EVs. PNPs have advantages of high biocompatibility, biodegradability, and ease of chemical modification (98). Chitosan, a natural cationic polysaccharide, is also used as a polymer scaffold for delivery of antagomirs (99). PNPs are often constructed as dendrimers, hyperbranched structures with high ASO-loading capacity along with the potential to incorporate ligands that target pNP delivery to specific cell types such as cardiomyocytes (100). However, use of pNPs to deliver ASOs *in vivo* is limited by the complex procedures that are required for their synthesis and large-scale production and by limited knowledge of their pharmacokinetic and pharmacodynamic properties (101).

3.6 Route of administration

The efficacy of ASO therapies—whether delivered by viral or nonviral vectors—can be enhanced by selection of an optimal route of administration. As mentioned above, parenteral injection—either intravenous or subcutaneous—is the most common route for administering ASOs (41), with similar *in vivo* biodistribution with both routes (Fig. 1D) (102). However, catheters can be used to improve delivery of antagomirs to the heart (103). For example, Hinkel et al. used both antegrade and retrograde catheter-based intracoronary approaches to deliver anti-miR-92a to cardiomyocytes. Both approaches increased delivery to ischemic myocardium compared to intravenous injection (103). ASOs may also be directly injected to the myocardium or embedded in hydrogel scaffolds (Fig. 1F) that are attached to the injured myocardium (104, 105). To improve ASO delivery to cells of the vessel wall, ASOs (either free or encapsulated, as well as ASO-expressing viral vectors) could be injected into the arterial adventitia (Fig. 1E). Adventitial injection has been used to deliver viral vectors or non-encapsulated ASOs to the artery wall (106, 107). Theoretically, transduction of vascular cells or cardiomyocytes with gene-transfer vectors expressing exosome-targeted antagomirs could exploit the natural process of exosome-mediated cell-cell communication and deliver antagomirs to cells throughout the vessel wall or the heart (108).

In summary, numerous non-viral and viral vector-mediated approaches for *in vivo* antagomir delivery have shown promise in preclinical models. Delivery of antagomirs to hepatocytes using the non-viral ASGR1-targeted system seems clinically viable; however, no delivery systems yet achieve comparable results with cardiomyocytes or vascular cells. Ideal delivery systems would incorporate ligands or cis-acting regulators of transcription that ensure efficient and cell-type-specific delivery and expression. If a viral vector is used, it should have low immunogenicity, a low prevalence of pre-existing immunity, and—to avoid genotoxicity—it should be non-integrating. Both viral and non-viral vectors must be biocompatible with substantial bioavailability. An optimal delivery platform would be modifiable to permit both transient and durable antagomir expression/persistence, adjusted according to therapeutic needs. Moreover, the potential for subcutaneous versus intravenous administration expedites clinical implementation, with oral administration a distant goal. Finally, to address regulatory and financial concerns, an antagomir-delivery platform must be amenable to production methods that are standardized, scalable, and economically viable.

4. Use of miRNA-targeted ASOs for treatment of cardiovascular diseases

Here we discuss several illustrative examples of recent preclinical and clinical studies that show the promise of antagomirs for treating three major CVDs (Table 3): i) atherosclerosis, ii) MI and its complications, and iii) cardiac hypertrophy, including hypertrophy driven by hypertension. These examples may not be comprehensive, and we regret any significant omissions.

4.1 Atherosclerosis

Preclinical data suggest that miR-33a has several pathogenic activities within atherosclerotic plaques including suppression of cholesterol efflux, inhibition of lipid droplet catabolism, reduction of efferocytosis, and maintenance of a proinflammatory immune cell profile (16, 109, 110). Accordingly, targeting of miR-33a with ASOs is atheroprotective in both mice and non-human primates. Systemic administration of anti-miR-33a increases circulating high-density lipoprotein-cholesterol in mice and non-human primates and reduces the size and lipid content of murine atherosclerotic plaques (111, 112). However, long-term systemic delivery of anti-miR-33a in mice fed a high-fat diet alters hepatic metabolism, leading to hypertriglyceridemia and hepatic steatosis (113). These toxic effects of hepatic miR-33a inhibition highlight the need for a delivery system that could target anti-miR-33a to plaque cells and minimize its activity at other sites.

As an initial step in developing vessel-wall-targeted miR33a inhibition, our group generated a helper-dependent adenoviral vector encoding an anti-miR33a antagomir fused to the X-motif (see above). We added the X-motif to promote loading of the antagomir into exosomes that are released from transduced cells. Medium conditioned by transduced EC contained anti-miR-33a-loaded exosomes and unconcentrated conditioned medium efficiently delivered anti-miR-33a to macrophages and VSMCs *in vitro*, decreasing miR-33a levels, increasing ABCA1 protein, and promoting ApoAI-mediated cholesterol efflux in both of these cell types (108). We anticipate that exosomes released by transduced arterial endothelial cells (which are efficiently transduced by adenoviral vectors *in vivo* using an intraluminal dwell approach, in which vectors are incubated in the lumen of a segment of blood vessel that is transiently isolated from the circulation with ligatures or clamps) (114, 115) would transfer the antagomir to neighboring vascular cells including macrophages and VSMCs, stimulating cholesterol efflux and reducing plaque size and lipid content, while favorably altering the plaque immune cell population (108). Use of vectors that achieve durable transgene expression (e.g., helper-dependent Ad, AAV, or lentivirus) could provide long-term protection from atherosclerosis. Recently, Li et al. developed anti-miR-33a-loaded polymer NPs coated with an $\alpha_v\beta_3$ integrin-binding ligand designed to target the NPs to plaque ECs and macrophages (116). Analysis of atherosclerotic mice after 8 weeks of injections showed that the NPs accumulated within the aortic plaque, reduced lesion size, and promoted an anti-inflammatory macrophage phenotype. Importantly, no long-term adverse effects were reported in blood, liver, kidneys, spleen, and lungs (116). This approach is promising, but the need for weekly injections and issues of cost are barriers to clinical translation.

Expression of the atherogenic miR-712 is upregulated in ECs by disturbed blood flow. MiR-712 downregulates expression of the metalloprotease inhibitor Timp3, leading to increased activity of TIMP3 targets including matrix metalloproteinases and disintegrin and metalloproteases as well as increased endothelial inflammation and permeability (117). Subcutaneous injection of a miR-712 antagomir in mice prevented these atherogenic effects of miR-712, leading to reduced aortic atherosclerosis. However, this antagomir also repressed miR-712 expression in non-vascular tissues (e.g., spleen), potentially causing off-target effects (117). To avoid off-target effects outside the vasculature, Kheirolomoom et al. constructed an LNA-modified anti-miR-712 with a phosphorothioate backbone, encapsulated the antagomirs in cationic lipid NPs coupled to a Vcam1-targeting peptide, and injected the NPs intravenously in mice (118). The NPs were efficiently delivered to aortic ECs, de-repressed expression of Timp3, and reduced atherosclerosis. Importantly, the effective dose of the encapsulated antagomir was lower than the effective dose of the naked antagomir (118). Moreover, injection of the NPs did not alter miR-712 levels in non-vascular tissues including spleen, liver, kidneys, and bone marrow. These data suggest that encapsulation of antagomirs in targeted NPs may reduce systemic toxicity (118).

4.2 Myocardial infarction (MI) and its complications

MiR-1 is a muscle-enriched miRNA that is upregulated in ischemic human and murine hearts and impairs cardiac function after MI by lowering the expression of proteins involved in ion trafficking (e.g., Kcnj2 and Gja1) and apoptosis suppression (e.g., Prkce and Bcl2). Accordingly, elevated miR-1 expression after MI promotes arrhythmias and cardiomyocyte death (24, 119). Intramyocardial injection of anti-miR-1 antagomirs reduced the incidence of ventricular arrhythmias in a rat MI model, and systemic injection of anti-miR-1 decreased infarct size in a mouse model (24, 119). Unfortunately, off-target effects in other tissues expressing miR-1, particularly skeletal muscle, were not evaluated. To improve targeting of ischemic myocardium and prevent off-target effects, Liu et al. encapsulated anti-miR-1 in liposomes coated with anti-cardiac troponin I antibody (120). Intravenous injection of the liposomes after experimental MI in rats efficiently targeted the ischemic heart, upregulated the ion channel proteins Kcnj2 and Gja1 and reduced arrhythmias. Importantly, the encapsulated antagomirs had low cytotoxicity *in vitro* and were taken up preferentially in ischemic myocardium versus skeletal muscle after intravenous injection (120). Xue et al. used PEGylated dendrimers coated with an anti-angiotensin type 1 receptor-binding peptide to target anti-miR-1 to hypoxic murine myocardium after intravenous injection. Anti-miR-1 reduced infarct size and prevented cardiomyocyte apoptosis, potentially by de-repression of Prkce and Bcl2 (100). The dendrimers had low cytotoxicity *in vitro*, although their distribution and potential side effects in non-cardiac tissues were not fully evaluated (100).

MiR-92a represses expression of several pro-angiogenic factors (e.g., ITGA5 and KLF4), thereby limiting revascularization of ischemic myocardium. Accordingly, suppression of miR-92a could promote myocardial revascularization (121, 122). Preclinical studies show that miR-92a enhances angiogenesis and tissue vascularization in murine MI and hindlimb ischemia models, in part by de-repressing Itga5 (122). In addition, Bellera et al. injected PLGA NP-encapsulated anti-miR-92a into coronary arteries of pigs with experimental myocardial ischemia and found that it repressed miR-92a for at least 10 days (123).

Moreover, these NPs were preferentially retained in the vessels of the injured myocardium and promoted angiogenesis. No NP accumulation was detected in other tissues, including liver, spleen, and lungs. Higher doses of NPs were associated with adverse effects on the heart; however, long-term effects were not evaluated (123). Fujita et al. delivered anti-miR-92a to infarcted rat hearts by direct attachment of a gelatin hydrogel microsphere sheet impregnated with anti-miR-92a. This novel approach promoted angiogenesis and cardiomyogenesis in the infarct border zone, leading to improved left ventricular function (104). These studies prompted initiation of the first clinical study that tested angiogenic antagomirs in humans (53). In this study, intravenous injection of an LNA-modified anti-miR-92a reduced plasma miR-92a levels and increased plasma levels of proteins encoded by miR-92a target genes, including the pro-angiogenic ITGA5.

The role of miR-21 in MI is controversial (124); however, several groups have identified miR-21 as a target for interventions that aim to decrease cardiac fibrosis after MI. These studies are based on the premise that miR-21 promotes cardiac fibrosis by at least 2 pathways: (1) inhibition of Smad7, which in turn de-represses pro-fibrotic TGF-ß signaling via Smad2/3 (125), and (2) inhibition of Pten, leading to increased Akt1 phosphorylation and upregulation of Mmp2, which helps drive cardiac fibrosis (126). Kang et al. loaded peripheral blood-derived EVs with anti-miR-21 and incubated the EVs with mouse and rat cardiomyocyte cell lines. Cells incubated with the EVs had lower levels of miR-21 along with higher levels of miR-21 targets (e.g., Smad7 and Pten) (105). Moreover, intramyocardial injection of these EVs in a mouse MI model significantly reduced cardiac fibrosis (i.e., decreased cardiac collagen types I and III) and increased myocardial levels of the miR-21 targets Smad7 and Pten (105).

Let-7i-5p, one of the most abundant miRNAs in the heart, inhibits cardiomyocyte proliferation by repressing the cell cycle regulators E2f2 and Ccnd2 (73). Accordingly, suppression of let-7i-5p could promote cardiac regeneration after MI. Hu et al. injected an AAV9 vector encoding an anti-let-7i-5p antagomir into infarcted mouse hearts and observed increased levels of myocardial E2f2 and Ccnd2, enhanced cardiomyocyte proliferation in the infarct border zone, and reduced infarct size (73).

4.3 Cardiac hypertrophy

Several miRNAs play roles in regulating cardiomyocyte growth and hypertrophy. For example, miR-20b is increased in hypertrophic hearts of hypertensive mice and promotes cardiac hypertrophy by downregulating Mfn2. Mfn2 regulates Ca^{2+} trafficking between the sarcoplasmic reticulum and mitochondria, and reduced cardiomyocyte Mfn2 levels lead to increased cytoplasmic Ca^{2+} and activation of calcium-induced hypertrophic signaling pathways (72). MiR-20b may also promote cardiac hypertrophy indirectly by stimulating proliferation and migration of VSMCs and increasing their synthesis of collagen and fibronectin leading to arterial stiffness and hypertension. The actions of miR-20b in VSMCs likely occur via inhibition of Magi3, although the precise mechanism is uncertain (127).

Several preclinical studies show that antagomirs can prevent cardiac hypertrophy. To prevent hypertrophy in hypertensive mice, Qiu et al. constructed an AAV9 vector expressing anti-miR-20b and injected it intravenously. AAV9-anti-miR-20b restored cardiac Mfn2

expression, decreased left ventricle wall thickness, and lowered levels of plasma markers of cardiac hypertrophy [i.e., atrial natriuretic peptide (Nppa) and brain natriuretic peptide (Nppb)] (72). Unfortunately, no data regarding potential adverse effects of the anti-miR-20b-expressing vector were reported. MiR-23a and miR-182 also promote cardiac hypertrophy, and both have been successfully targeted by antagomirs. MiR-23a is upregulated in cardiomyocytes by calcineurin and promotes cardiac hypertrophy by inhibiting the anti-hypertrophic protein Ring1 (128). Accordingly, knockdown of miR-23a by intravenously injected 2'-O-methyl modified antagomirs attenuated hypertension-induced hypertrophy in mice (128). Similarly, intravenous injection of cationic liposome-like NPs coated with anti-miR-23a downregulated miR-23a expression in mouse myocardium, reduced cardiac hypertrophy, and improved cardiac function (129). However, biodistribution and adverse effects of these NPs were not analyzed.

MiR-182 targets both the anti-hypertrophic Foxo3 transcription factor and the Bcat2 enzyme (inhibitors of the calcineurin and AKT/mTORC1 pathways, respectively), leading to accelerated cardiomyocyte growth (130–132). To block the hypertrophy-promoting effects of miR-182, Zhi et al. loaded cholesterol-coated polymer NPs with anti-miR-182 ASOs and injected the NPs intravenously in mice. The NPs efficiently delivered the ASOs to cardiomyocytes *in vitro* and to murine hearts *in vivo*, increasing myocardial Foxo3, reducing cardiomyocyte size, and improving cardiac function (132). Importantly, this delivery system had low cytotoxicity *in vitro*, and did not alter the histology of other organs such as liver and kidney when injected into mice. However, these promising findings should be interpreted with caution because inhibition of miR-182 in murine hearts was not verified, and other reports indicate that miR-182 expression is not upregulated in mouse myocardium subjected to pressure overload (131).

5. Conclusions and future perspectives

MiRNAs play fundamental roles in the pathogenesis of multiple CVDs. Antagomirs have emerged as a promising therapeutic strategy to inhibit miRNA activity and show substantial promise in preclinical models. To date, two antagomirs (i.e., anti-miR-92a and anti-miR-132) have been used in clinical trials for treatment of patients with CVDs (53, 54). Anti-miR-132 has advanced to a phase II clinical trial for treating MI patients with reduced left ventricular ejection fraction. However, despite significant improvements in antagomir chemistry and targeting, advancement of miRNA-targeted antagomirs into the clinic still faces numerous challenges. Inefficient targeting of cardiovascular cells (i.e., cardiomyocytes, endothelial cells, and smooth muscle cells) and accumulation of antagomirs in non-targeted organs reduces efficacy and increases toxicity *in vivo*. Encapsulation of antagomirs into targeted delivery systems (or expression of antagomirs by tissue-tropic viral vectors) has enhanced the therapeutic potential of antagomirs, reducing delivery to non-targeted cell types and improving both efficacy and safety in preclinical models of CVDs. However, scalability and cost are persistent challenges for both viral and non-viral vector systems. Further work is needed to improve the pharmacokinetic and pharmacodynamic properties of viral as well as non-viral vectors that are used to deliver antagomirs, for example by selecting optimal dosing and administration routes, by further improving targeting of specific cardiovascular cell types and by decreasing immunogenicity. Finally, approaches

that exploit endogenous cell-cell communication mechanisms (e.g., exosomes) should be further explored.

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Highlights

1. MicroRNAs (miRNAs) drive the onset and/or progression of multiple cardiovascular diseases.
2. MiRNA-targeting antisense oligonucleotides, or antagomirs, represent a promising therapeutic approach to precisely inhibit miRNAs.
3. Targeting systems (either viral or non-viral) enhance delivery of antagomirs to cardiac and vascular cells, while decreasing systemic exposure.

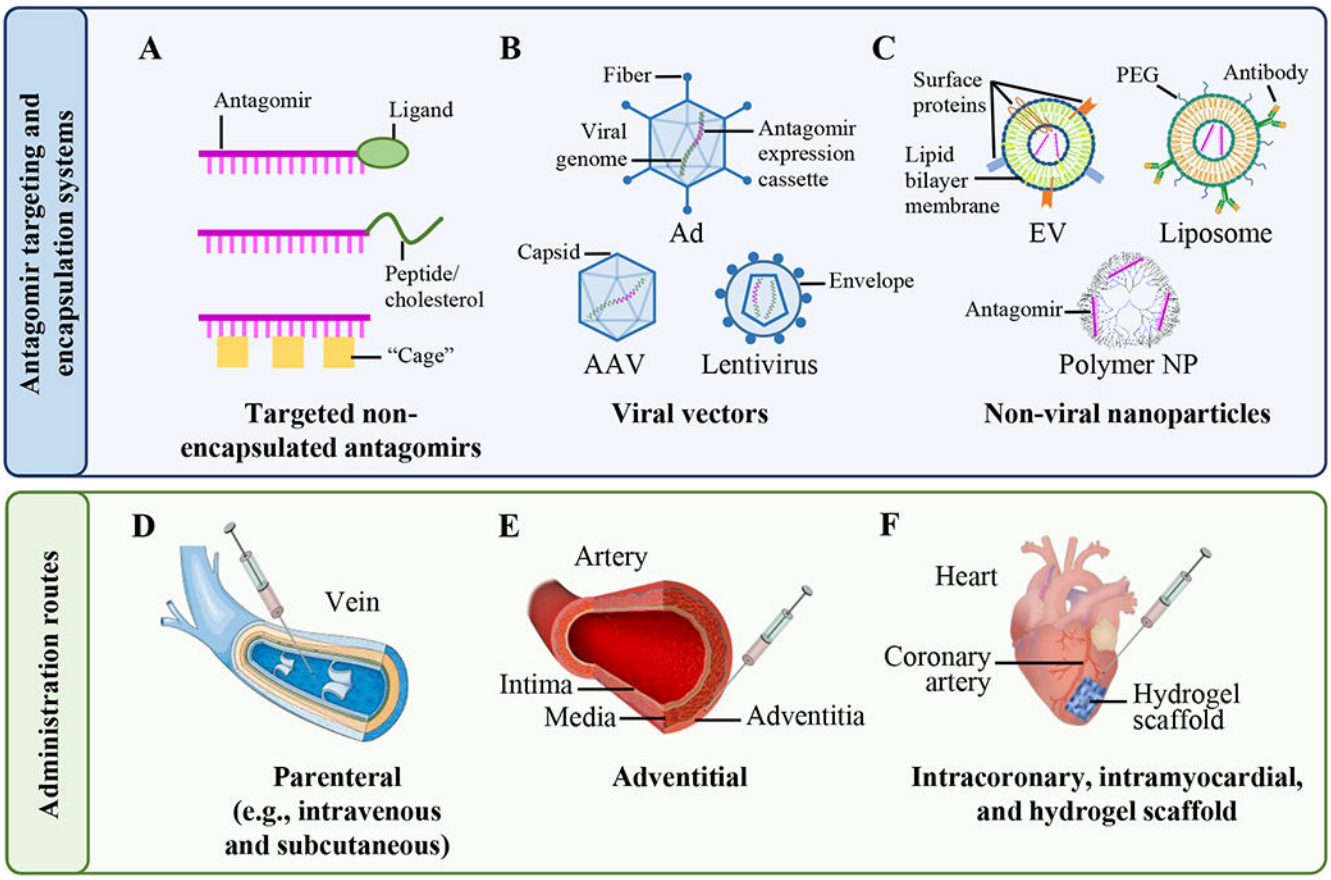

Figure 1.

Schematic representation of targeted antagomir delivery systems and administration routes. (Upper panel): Antagomirs (pink) are administered as non-encapsulated ASOs (A), are expressed from viral vectors (B), or are delivered by non-viral nanoparticle (NP) carriers (C). Some NP carriers can be loaded with plasmids (not shown) that express the antagomirs after cell entry. (A) Non-encapsulated antagomirs can be linked to functional groups such as ligands (e.g., N-acetylgalactosamine) that bind specific cell surface receptors or to cholesterol or cell-permeable peptides that enhance cell entry. Alternatively, photolabile "cages" can be linked to the antagomir nucleobases, allowing site-specific activation of the antagomir by illumination. (B) Antagomirs can also be expressed from viral vectors, typically adenoviruses (Ad), adeno-associated viruses (AAV), and lentiviruses (LV). In these cases, an expression cassette containing the antagomir sequence (pink) is inserted into the viral genome, potentially allowing long-term antagomir expression from within transduced cells. (C) Synthetic antagomirs (pink) or antagomir-encoding plasmids (not shown) can be incorporated within lipid-based NPs (e.g., extracellular vesicles (EVs), liposomes, or polymer-based NPs). Lower panel: Antagomirs are commonly administered via intravenous injection (D), subcutaneously (not shown), or by injection into the adventitia (E). Antagomirs can also be injected directly into the myocardium or applied to the epicardium using hydrogel scaffolds (F). Intracoronary administration (not shown) is also

used to deliver antagomirs to the myocardium. This figure was partly generated using Servier Medical Art.

Table 1.

Summary of miRNAs involved in CVDs.

CVD	miRNA	Target genes	Biological role of miRNA	Ref.
Atherosclerosis	miR-10a	MAP3K7 and BTRC	Reduced expression of endothelial adhesion molecules and proinflammatory cytokines	12
	miR-126	Dlk1	Enhanced proliferation of endothelial cells	15
	miR-155	Hbp1	Increased lipid uptake, synthesis of ROS, and foam cell formation	17
	miR-33a	ABCA1	Reduced cholesterol efflux and increased foam cell formation	18
	miR-145	KLF5	Prevents neointima formation	19
	miR-362	ADAMTS1	Prevents vascular smooth muscle cell proliferation and migration into the intima	22
Myocardial infarction (MI)	miR-1	Kcnj2 and Gja1	Decreased cardiac conductivity and induction of arrhythmias	24
	miR-433	Azin1	Activates TGF-β signaling to induce cardiac fibrosis	25
	miR-24	Gata2 and Pak4	Induction of apoptosis of endothelial cells and reduced neovascularization of the ischemic myocardium	26
	miR-134	Kdm2a	Repression of myocardial angiogenesis after MI	27
Hypertension and hypertensive heart disease	miR-181a and miR-663	REN	Downregulation may lead to higher renin expression in the renal cortex of hypertensive patients	29
	miR-143	ACE2	Downregulation may enhance ACE2 expression in the arteries of hypertensive patients	30
	miR-26	Gata4	Prevents pressure overload-induced cardiac hypertrophy	31
	miR-217	Pten	Promotes cardiac hypertrophy via AKT pathway activation	32
	miR-214	Sirt3	Enhances mitochondrial dysfunction and cardiac hypertrophy	33

CVD: cardiovascular disease; ROS: reactive oxygen species; MI: myocardial infarction

Table 2.

Advantages and disadvantages of ASO delivery systems.

Delivery system		Advantages	Limitations
Viral	Adenovirus (Ad)	• High packaging capacity (~37 kb) • High transduction rate of cardiac and vascular cells • Capsid modifications may enhance tropism towards cardiac and vascular cells • Non-integrative	• Strong inflammatory response (lower in third-generation Ads) • Presence of neutralizing antibodies • Inefficient targeting of cardiac and vascular cells
	Adeno-associated virus (AAV)	• Native tropism towards the cardiomyocytes (AAV1, 6, 8 and 9) and vascular wall cells (AAV1 and 5) • Capsid modifications may enhance tropism towards cardiovascular cells • Sufficient packaging capacity for antagomirs (~4.7 kb) • Mostly non-integrative	• Inflammatory response • Presence of neutralizing antibodies • Inefficient targeting of vascular cells
	Lentivirus (LV)	• High packaging capacity (~10 kb) • Long-term transgene expression • Low immunogenicity • Low prevalence of neutralizing antibodies	• Potential insertional oncogenesis (for integrating LV vectors) • Limited *in vivo* cardiovascular cell transduction, although pseudotyping may enhance tropism towards cardiovascular cells
Non-viral	Liposomes	• High loading capacity • Low immunogenicity • Allow functionalization	• Toxicity of cationic liposomes • Transient transgene expression
	EVs	• Highly biocompatible • Efficient cellular uptake • Readily scalable production • Allow functionalization	• Contain complex molecular cargo in addition to antagomirs • Limited batch-to-batch reproducibility
	Polymer NPs	• High loading capacity (especially dendrimers) • Biocompatibility (e.g., PLGA or chitosan) • Allow functionalization	• Toxicity of cationic polymers. • Limited knowledge of the PK/PD properties

NP: nanoparticle; EV: extracellular vesicle; PLGA: poly(lactic-co-glycolic acid); PK/PD: pharmacokinetic/pharmacodynamic.

Table 3.

Delivery of antagomirs in preclinical models of CVDs.

Cardiovascular disease (species)	Targeting approach and delivery system (route)	Target miRNA	Findings	Ref.
Atherosclerosis (mouse)	Anti-$\alpha_v\beta_3$ integrin polymer NPs (IV)	miR-33a	Reduced lesion size and increased M2 macrophages	116
Atherosclerosis (mouse)	Lentivirus (RO)	miR-33a	Increased hepatic ABCA1 protein and increased plasma HDL	110
Atherosclerosis (human and bovine cells)	Ad (*in vitro* study)	miR-33a	Increased ABCA1 protein and ApoA1-mediated cholesterol efflux	108
Atherosclerosis (mouse)	Anti-VCAM1 liposomes (IV)	miR-712	Reduced lesion size and Timp3 expression	118
MI (rat)	Anti-cardiac troponin I liposomes (IV)	miR-1	Repressed arrhythmias	120
MI (mouse)	Anti-AT1R dendrimers (IV)	miR-1	Reduced apoptotic cells, fibrosis, and infarct size	100
MI (pig)	Polymer NP (IC)	miR-92a	Enhanced angiogenesis	123
MI (rat)	Gelatin hydrogel microsphere sheet (local device)	miR-92a	Enhanced angiogenesis and cardiomyogenesis, reduced infarct size, and improved left ventricle function	104
MI (mouse)	EVs (IM)	miR-21	Reduced fibrosis and improved cardiac function	105
MI (mouse)	AAV9 (IM)	let-7i-5p	Reduced infarct size and enhanced cardiomyocyte proliferation	73
Hypertension (rat)	LV (IV)	miR-20b	Reduced blood pressure, artery wall thickness, and vascular fibrosis	127
Cardiac hypertrophy (mouse)	AAV9 (IV)	miR-20b	Decreased left ventricle thickness	72
Cardiac hypertrophy (mouse)	Liposome-like NP (IV)	miR-23a	Reduced left ventricle thickness and improved cardiac function	129
Cardiac hypertrophy (mouse)	Cholesterol-coated polymer NP (IV)	miR-182	Repressed cardiomyocyte growth and improved cardiac function	132

AT1R: angiotensin II type 1 receptor; AAV: adeno-associated virus; Ad: adenovirus; CVD: cardiovascular disease; NP: nanoparticle; VCAM1: vascular cell adhesion molecule 1; IC: intracoronary; IV: intravenous; RO: retro-orbital; IM: intramyocardial; EVs: extracellular vesicles; MI: myocardial infarction.