Abstract: Understanding of the roles of noncoding RNAs (ncRNAs) within complex organisms has fundamentally changed. It is increasingly possible to use ncRNAs as diagnostic and therapeutic tools in medicine. Regarding disease pathogenesis, it has become evident that confinement to the analysis of protein-coding regions of the human genome is insufficient because ncRNA variants have been associated with important human diseases. Thus, inclusion of noncoding genomic elements in pathogenetic studies and their consideration as therapeutic targets is warranted. We consider aspects of the evolutionary and discovery history of ncRNAs, as far as they are relevant for the identification and selection of ncRNAs with likely therapeutic potential. Novel therapeutic strategies are based on ncRNAs, and we discuss here RNA interference as a highly versatile tool for gene silencing. RNA interference-mediating RNAs are small, but only parts of a far larger spectrum encompassing ncRNAs up to many kilobasepairs in size. We discuss therapeutic options in cardiovascular medicine offered by ncRNAs and key issues to be solved before clinical translation. Convergence of multiple technical advances is highlighted as a prerequisite for the translational progress achieved in recent years. Regarding safety, we review properties of RNA therapeutics, which may immunologically distinguish them from their endogenous counterparts, all of which underwent sophisticated evolutionary adaptation to specific biological contexts. Although our understanding of the noncoding human genome is only fragmentary to date, it is already feasible to develop RNA interference against a rapidly broadening spectrum of therapeutic targets and to translate this to the clinical setting under certain restrictions. (Circ Res. 2013;113:588-602.)

Key Words: cardiovascular diseases • genetic therapy • immunity, innate • nanoparticles • RNA interference • RNA, long untranslated • RNA, short untranslated

Classical studies several decades ago have already shown the presence of large amounts of RNAs that are transcribed but do not encode proteins. Only part of these noncoding RNAs (ncRNAs) could later be explained by mRNA splicing, or was specifically involved in the translation machinery and its regulation (eg, transfer RNAs [tRNAs], ribosomal RNAs), whereas the overwhelming majority remained functionally cryptic for a long time. Rather recent studies then led to the discovery of entire new classes of small RNAs, generated not only by novel biosynthetic pathways and mediating regulatory functions (eg, miRs [microRNA]), but also of unusually large long ncRNAs (lncRNAs) of still uncertain functional significance. Cumulative evidence derived from multiple genetic, biochemical, and other experimental and clinical studies during the past decade strongly suggests a central role of ncRNAs in the genetic programming of complex organisms, during their development and in health and disease. The observation of a steeply increasing fraction of ncRNAs in the genome during evolution, from simple to highly complex organisms, in contrast to an only modest increase in the number of protein-coding genes, is consistent with the assumption of an overwhelming role of the ncRNA species in higher organisms. Regarding disease pathogenesis, it has become evident from the Encyclopedia of DNA Elements (ENCODE) project that confinement to the analysis of protein-coding regions of the human genome is inadequate because many noncoding variants are associated with important human diseases. Inclusion of the noncoding genomic elements in pathogenetic studies seems mandatory and approach is comprehensive transcriptome mapping encompassing protein-coding genes, as well as small and large ncRNAs.

Importantly, ncRNA can be both tools (eg, short interfering RNAs [siRNAs]) and targets (eg, lncRNAs) of novel therapeutic strategies. Thus, RNA interference (RNAi) may be considered as a highly versatile general-purpose tool for the silencing of not only specific single protein-encoding genes...
Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAV</td>
<td>adeno-associated virus</td>
</tr>
<tr>
<td>ASO</td>
<td>antisense oligonucleotide</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double-stranded RNA</td>
</tr>
<tr>
<td>IncRNA</td>
<td>long noncoding RNA</td>
</tr>
<tr>
<td>MALAT1</td>
<td>metastasis-associated lung adenocarcinoma transcript 1 (synonymous: NEAT2)</td>
</tr>
<tr>
<td>MI</td>
<td>myocardial infarction</td>
</tr>
<tr>
<td>MIAT</td>
<td>myocardial infarction-associated transcript</td>
</tr>
<tr>
<td>miR</td>
<td>microRNA</td>
</tr>
<tr>
<td>ncRNA</td>
<td>noncoding RNA</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RNA-seq</td>
<td>RNA sequencing</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>short interfering RNA</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
</tbody>
</table>

via mRNA, but also against non–protein-coding RNA targets. Recent studies have demonstrated therapeutic potential of organ-targeted RNAi on the basis of viral vectors or synthetic RNA, and developed therapeutic strategies on the basis of modulation of miR functions. Both RNAi-mediating molecules and miRs are of small size, but only parts of a far larger spectrum of ncRNAs arising from the human genome up to thousands of base pairs in size. lncRNAs range from 200 nt to >100 kb in length, excluding infrastructural or regulatory RNAs (tRNAs, miRs, sRNAs, etc). Transcriptional surveys in mammals suggest that lncRNAs may outnumber protein-coding genes, but biological functions have been assigned only to a minute fraction of them. Overall, only a small part of the noncoding human genome has been investigated and explored with regard to possible therapeutic implications for cardiovascular medicine.

RNAi: The Broadening Target Spectrum

RNA has been the subject of landmark discoveries with catalytic RNA in 1989, splicing in 1993, RNAi in 2006, ribosomal structure in 2009, and others, which have led to a radical reassessment of RNA functions during evolution and in human diseases. Only 1.2% of the human genome encodes for proteins and the 20,000 recognized human proteins are similar in number with orthologous functional overlaps to those in animals down to the simple nematode worm. This revolutionary insight has triggered extensive research into the functions of the noncoding genome and also led to an extended theory of evolution. Certain very ancient ncRNA systems were discovered in the 1990s, this revolutionized thinking about RNA functions and introduced a huge number of novel players involved in cellular functions and disease pathogenesis. Although this first expansion in the number of biologically and pathogenetically important RNA species already triggered a huge number of important and highly innovative studies during the past 2 decades, it seems clear that a second expansion arrived with the discovery of a huge number of lncRNAs (and processing products) about which we do not know much more than about miRs when they were discovered. Again, technological progress plays a key role in the further elucidation of highly complex new systems. Knowledge of the mechanisms by which lncRNAs act is sparse, and only few well-characterized lncRNAs have to date given clues about their cellular roles. Current evidence strongly supports pervasive transcription of mammalian genomes, but the functional biological significance of many “Dark Matter” transcripts (novel RNAs of unknown function) remains enigmatic. Notwithstanding these limitations, first functional and mechanistic insights have begun to emerge and warrant their investigation regarding pathogenetic and therapeutic implications, if they are found to be dysregulated in certain diseases. As discussed by important recent reviews and below, there is sufficient evidence to investigate them also in certain cardiovascular disorders.

Technological Progress as Key Driving Force

Novel technologies were importantly involved in the discovery of multiple classes of small RNAs, including miRs, snRNAs, snoRNAs, siRNAs, piRNAs, tRNAs, spliRNAs, sdRNAs, and others, and of lncRNAs thousands of which have been identified in intergenic regions alone. On the one hand, this immense expansion of regulatory RNAs not encoding for proteins already requires a huge amount of research to identify biological function of individual RNAs and to characterize their molecular mechanisms of action. On the other hand, there are arguments to suggest that traditional extraction of isolated functions, for example, gain/loss-of-function models or biochemical studies in vitro, may not be suitable to clarify their overall biological role because highly complex interaction networks are involved. An interesting recent theory aims at unified understanding of the extremely broad
spectrum of RNA species. This hypothesis attempts to explain how mRNAs, transcribed pseudogenes, and lncRNAs talk to each other using miR response elements as letters of a new language. The authors propose that this competing endogenous RNA activity forms a large regulatory network across the transcriptome, fundamentally expanding the functional genetic information in the human genome and playing important roles in pathological conditions.

Toward Comprehensive Human Transcriptome Mapping

When considering therapeutic targets for RNAi, the obvious candidates are well-characterized protein-encoding genes with already established cardiovascular pathogenic relevance, in particular if no conventional inhibitory drug is available for the coded protein. As reviewed below, however, there is already sufficient evidence to warrant also the evaluation of several lncRNAs dysregulated in cardiovascular diseases as novel targets for RNAi. We discuss which selection criteria one might use to identify lncRNAs of therapeutic relevance in the cardiovascular system. Selection criteria are needed, indeed, because of the vast number of noncoding transcripts, which are identified by state-of-the-art technologies in normal and diseased hearts. Until recently, transcriptome mapping based on microarrays failed to capture the full catalogue of transcripts and their variations. Advances in sequencing technology now allow, however, the reconstruction of truly complete transcriptomes by deep RNA sequencing (RNA-seq) in the absence of a reference genome. Furthermore, comprehensive RNA quantification is now possible from very small amounts of cellular material (eg, from cardiovascular bioptic material taken from patients during diagnostic procedures). Whereas microarrays mainly detect transcripts with more or less well-defined functions; RNA-seq includes vast numbers of transcript sequences of unknown functional relevance and context. In the cardiovascular field, RNA-seq has revealed cardiac transcriptomic signatures for physiological and pathological hypertrophy, including not only overall gene expression analysis, but also individual transcript isoforms, novel spliced
exons, novel alternative terminal exons, novel transcript clusters, and IncRNAs.30

Broad Versatility of RNA Structures
Different from DNA, RNAs are carrying information not only in their linear sequences of nucleotides (primary structure), but also local nucleotide pairing creates secondary structures, for example, hairpins, and interaction among distantly located sequences create tertiary structures. In fact, this extreme structural versatility needs to be considered for RNAs as therapeutic tools (short hairpin RNAs [shRNAs], siRNAs, antisense oligonucleotides [ASOs], aptamers), as well as therapeutic targets (eg, IncRNAs). The use of computational prediction methods and recent experimental techniques to probe RNA structure by high-throughput sequencing has enabled genome-wide measurements of RNA structure and may in the future reveal a picture of the RNA structureome.31 The plethora of RNA types, sequences, and structures, which were created by evolution, is a treasure trove of potential therapeutic tools and targets. However, criteria need to be developed so that promising candidates for further in-depth mechanistic, pathogenic, and therapeutic studies can be selected.

RNAi: Versatile Principle for Silencing of Protein-Coding Genes and ncRNA Systems
Classification of Therapeutic RNAs
Regarding the plethora of possible therapeutic ncRNAs, one should distinguish RNAs addressing a single gene encoding a protein with well-characterized function, for example, shRNAs mediating classical RNAi, others addressing multiple protein-encoding genes, for example, miRs targeting multiple 3′-untranslated regions, IncRNAs interacting with multiple RNAs and RNA–protein complexes, and processing products from IncRNAs. Whereas all types may have therapeutic potential; current technologies do not permit therapeutic delivery of large RNA molecules in vivo, whereas the delivery of small RNAs mediating posttranscriptional gene silencing (PTGS) has made significant progress toward the clinic. In this context, it is of interest that recent work on IncRNA processing has revealed new biosynthetic pathways generating small RNAs from IncRNA precursors.32–34 It seems entirely possible that hitherto unknown common biosynthetic pathways generate novel classes of small RNAs from IncRNAs, in a similar way as the large spectrum of miRs is generated from prepro-miRs via Dicer and Drosha. Such novel classes of small ncRNAs could then be targeted by already available PTGS technologies, including RNAi.

Similarities and Differences Between RNAi and miRs
Regarding the mechanisms of action of ncRNAs, we first review mechanisms of PTGS dependent on small RNAs, including siRNAs, shRNAs, and miRs.35,36 Second, we discuss mechanisms beginning to emerge for IncRNAs, as well as first data on functions of IncRNA processing products. When the mechanisms of ncRNA actions are considered with the perspective of possible therapeutic application, one may distinguish different categories defined by their levels of specificity. When an RNAi trigger is chosen to silence a single target gene and its encoded protein specifically, the situation resembles traditional drug therapies with well-defined single targets, and also the concept of traditional gene therapy overexpressing single proteins. A second, less well-defined situation arises when miRs are either enhanced or suppressed because a single modulated miR influences the function of multiple genes and thus multiple encoded proteins. If the function of a single miR within a specific cell or organ has been well defined, the consequences of its modulation should nevertheless be predictable. As exemplified below, however, a single miR may exert grossly different actions dependent on cell type and pathogenic situation, so that miR modulation therapies require precise cellular targeting. IncRNA systems have even more complex and context-dependent mechanisms of action, so that therapeutic translation will certainly take time and probably the development of novel therapeutic tools.

Classical RNAi
Regarding RNAi, its original discovery had revealed that introduction of double-stranded RNA (dsRNA) into a cell can trigger a target sequence-specific gene silencing mechanism. Within the cell, the dsRNA is processed into ≈22-nt fragments by an evolutionary ancient, endogenous machinery. This endogenous RNAi was first discovered and analyzed in plants and nematodes, but later was found to occur also in higher organisms, including mammals.35 It was also observed that the silencing effect of dsRNA may be mimicked by chemically synthesized siRNA, and one may designate this as exogenous RNAi. The task to produce siRNAs in vivo was solved by designing vectors expressing shRNAs from RNA polymerase III promoters (U6, H1, 7SK), which are then endogenously processed to active siRNAs. In parallel with the study of RNAi, progress in understanding the cellular processing and function of miRs was achieved. RNAi and miR systems share most components of the silencing pathway (Figure 2). In the nucleus, the initial precursors of miRs designated as primary miRs are transcribed from their own genes, or from introns, by polymerase II or III, and processed by an enzyme complex (Microprocessor) consisting of Drosha, a polymerase III-type dsRNA-specific endonuclease, and its cofactor microprocessor complex subunit DGC8R8, to premiRs. In contrast, shRNAs are transcribed in the nucleus via polymerase III, from plasmids of viral vectors transfected/transduced into the cells. Both premiRs and shRNAs are exported from the nucleus to the cytoplasm by exportin-5, a nuclear membrane protein recognizing the 2-nucleotide overhang at the 3′-end of the premiRs or shRNAs, respectively. In the cytoplasm, both premiRs and shRNA are cleaved by Dicer, a polymerase III-type dsRNA endonuclease which associates with its cofactors transactivation response RNA-binding element protein and protein activator of PKR. The small RNA duplexes thus generated are then processed to the mature double-stranded miRs or siRNAs. One strand of the products represents the mature guide strand miR or siRNA bringing the Dicer–transactivation response RNA-binding protein–protein activator of PKR complex into contact with argonaute proteins, whereas the opposite passenger strand is discarded. The mature miRs and siRNAs then guide the resulting RNA-induced silencing complex complex to their respective target sites on
mRNAs. Perfect complementarity between a miR/shRNA and its mRNA target sites leads to endonuclease cleavage of this mRNA by argonaute-2, whereas imperfect complementarity between a miR and its target mRNA results in translational stop. Recent studies have elucidated subtle differences between the mechanism by which siRNAs and miRs lead to target gene silencing, by assigning distinct biochemical properties to different domains established by Argonaute: anchor, seed, central, 3′-supplementary, and tail region.

Largely Unexplored Spectrum lncRNA Mechanisms
The molecular mechanisms of lncRNA action are highly diverse and complex (Figure 3), and they operate not only in cis, but also in trans. Cis-acting lncRNAs are confined to their sites of synthesis and act locally on spatially linked genes on the same chromosome. Trans-acting lncRNAs may migrate from their site of synthesis and act genome wide, similar to transcription factors and small ncRNAs. First, lncRNAs function as molecular scaffolds by forming interaction interfaces with other RNAs, as well as proteins (e.g., polycomb group complexes, chromatin modifiers). Thus, metastasis-associated lung adenocarcinoma transcript 1 (synonymous: NEAT2; MALAT1) is involved in the ncRNA-dependent and Pc2 methylation–dependent gene relocation between nuclear structures and thus mediates gene activation programs. Second, lncRNAs generate regulatory RNA–protein complexes in the nucleus, and alternative IncRNA splicing change the interaction domains of the RNA altering the structure and resulting function of the protein complex. Thus, NEAT1 is important for a highly ordered spatial organization of paraspeckles and other nuclear bodies. Third, IncRNAs may act as a guide targeting a RNA–protein complex to specific genome regions or through RNA–RNA interactions linking otherwise not interacting protein complexes. Fourth, dynamic conformational changes of lncRNAs may act as biological switch in response to diverse cellular stimuli. lncRNAs can influence the transcriptional landscape in a comprehensive manner, from epigenetic regulation and chromatin remodeling to transcriptional and post-transcriptional control to protein metabolism. Beyond these functions of the long primary transcript, there may be a plethora of specific functions exerted by lncRNA processing products, some of which are rather small and likely to migrate between cell compartments possibly serving signaling functions. Our knowledge in this regard is very limited, however, and must await the outcome of (technically demanding) kinetic studies.

The recent discovery of tens of thousands of IncRNAs has temporarily overwhelmed the capacities to assign their functions and mechanisms of action. Nevertheless, important

**Figure 2. RNA interference (RNAi): versatile principle for silencing of protein-coding genes and noncoding RNA systems.** The broad spectrum of RNAs with therapeutic potential ranges from RNAi triggers to antisense oligonucleotides (ASOs), ribozymes, aptamers, and others. Among these RNA tools, we consider here those mediating RNAi, and as targets for RNAi we discuss protein-coding mRNAs and long noncoding RNAs (lncRNAs). Target silencing may be achieved using synthetic RNA molecules modified to achieve biostability and delivered by synthetic vehicles, for example, nanoparticles, or using viral vectors (preferentially organ-targeted and regulatable) with the capacity for long-term production of therapeutic RNA. It should be noted that all these RNA drugs act on delicately balanced endogenous systems where target genes have promoters and feedback mechanisms to ensure homeostasis, and all endogenous RNAs are immunologically inert, whereas synthetic and recombinant RNA drugs and their vectors may arise immune reactions, and dosage of RNA therapeutics needs to be adapted to requirements. Reprinted from Poller and Fechner with permission of the publisher. Ago indicates argonaute; DGCR8, microprocessor complex subunit DGCR8; POL, polymerase; and shRNA, short hairpin RNA.
pioneering work has led to functional insights for a distinct group of vertebrate-specific lncRNAs, MALAT1/NEAT2, NEAT1/MENε/β, and MI-associated transcript (MIAT)/Gomafu, which accumulate abundantly in the nucleus as RNA components of specific nuclear bodies.42 Animal models for MALAT1 and for NEAT1 have been developed and although they in the first place yielded apparently normal mice, impressive differences between normal and knockout animals were revealed under pathological conditions. As in other traditional gene knockout models, only pathogenic stress may reveal the true functional spectrum of lncRNAs, and studies of this type are to date very rare in the field. Functional investigation of lncRNA systems may also require nonclassical knockout technologies by which the lncRNA gene is not germ line deleted but silenced in the adult organism by RNAi.

RNAi: Classical Therapeutic Silencing of Protein-Coding Genes

Progress by Independent Synergistic Technological Developments

The therapeutic potential of strategies based on ncRNAs is critically dependent on a combination of technological developments which have converged from different areas of research. Pioneering work in the first era of gene therapy has focused on inherited diseases (eg, the hemophilias, viral diseases, and cancer). For a long time, no delivery systems for cardiovascular gene therapy were available, but recently breakthrough and successful clinical translational was also achieved in this area.3,51–55 One may consider the novel therapeutic approaches discussed in this review as a second era of gene therapy where gene silencing is intended, whereas in the first era the goal was the addition or substitution of protein-coding genes.43,51,56,57 Below we discuss analogies and differences between ncRNA therapy and classical gene therapy because recent breakthroughs in vector technology are of importance for both.55,58

Recent Developments in Cardiovascular RNAi

One example of the second era in the cardiovascular field is the combination of a highly efficient cardiotropic RNAi vector to silence the cardiac-expressed regulatory protein phospholamban, with a cardiotropic vector system based on adeno-associated virus (AAV), which has for the first time shown high efficacy of an RNAi therapeutic strategy in a cardiac disease.3 The AAV system enables long-term RNAi therapy by the continuous production of shRNAs from the long-term stable vector. Recently, nanoparticle-encapsulated synthetic siRNA was used for silencing of C-C chemokine receptor type 2, the chemokine receptor that governs inflammatory Ly-6C monocyte subset traffic. This approach reduced recruitment of Ly-6C monocytes, attenuated infarct inflammation and post–myocardial infarction (MI) left ventricular remodeling.4 Another important recent study introduced a strategy for in vivo RNAi imaging.59 RNAi targeted prolyl hydroxylase domain protein 2 to treat myocardial ischemia, and the shRNA sequence was...
followed by a separate hypoxia response element-containing promoter driving a firefly luciferase reporter gene. This construct allowed noninvasive monitoring of gene expression and was used to demonstrate that inhibition of prolyl hydroxylase domain protein 2 by RNAi improved angiogenesis and contractility in vitro and in vivo. RNAi has also been used in conjunction with cardiovascular stem cell therapy. One of the limitations of this approach is poor stem cell survival in the diseased microenvironment, and RNAi-based prolyl hydroxylase domain protein 2 silencing enhanced survival and paracrine function of transplanted adipose-derived stem cells in infarcted myocardium. Allele-specific RNAi as novel approach for the treatment of many autosomal-dominant-negative disorders rescued the long-QT syndrome phenotype in human-induced pluripotency stem cell cardiomyocytes. In the context of heart transplantation, RNAi has been used to induce alloimmune tolerance through silencing of toll-like receptor adaptors MyD88 and TIR-domain-containing adapter-inducing interferon-β. In vivo treatment of recipients with anti-MyD88 and anti-TIR-domain-containing adapter-inducing interferon-β siRNAs prolonged allograft survival. It should be emphasized that in addition to its direct use to improve the treatment of cardiovascular diseases (by delivery of therapeutic RNAi triggers in vivo), RNAi has also proven highly valuable for reverse genetics. It may first serve to identify novel functions of specific protein-coding genes and ncRNAs, and second to evaluate their potential as therapeutic targets. Cardiovascular examples include myotrophin, myocardin, activator protein-2α, Na+/Ca2+ exchanger, malic enzyme 1, cyclophilin-D, or chymase in the context of plaque stability in atherosclerosis. These studies exemplify valuable indirect applications of RNAi to improve therapy via the identification and validation of putative therapeutic targets for which no conventional inhibitory drugs are available. A large number of important studies have used RNAi system for in vitro and in vivo ablation of specific protein-coding, as well as noncoding gene functions, and this approach has also been systemically used in genome-wide functional scans.

**Characteristic Properties of RNA Therapeutics**

Although our ability to understand the impact of small ncRNA in disease states is preliminary, and our ability to target them in vivo is limited; several companies have already been formed with the goal of developing RNA therapeutics. Similar to other recent fields in human therapy research, early enthusiasm has been followed by a realistic assessment of the key steps that are prerequisite before successful clinical translation. In the field of RNA therapeutics these are (Figure 4): (1) specificity of the therapeutic RNA for the target, (2) stability or continuous in situ production of the therapeutic RNA or the ability to reapply if needed, (3) targeting to the correct cells by the use of appropriate vectors and other methods, (4) side effects induced by the therapeutic RNA itself or the delivery system need to be minimized. In this context, innate immune response to synthetic RNA is an important novel aspect, (5) regulatability of vector systems for therapeutic RNAs. Because of the peculiar biochemical properties of small RNA molecules, clinical translation is more demanding than with small molecule drugs or proteins. Overall, RNA therapeutics (Figure 4) may be chemically synthesized nucleic acids (ranging from Ribozymes to Aptamers/Spiegel mers) delivered using synthetic chemicals, or produced by recombinant viral vectors thus taking advantage of their biological targeting properties. Viral vectors have the capacity to act as long-term productive and, ideally, also regulatable RNA drug factories, whereas chemical synthesis allows introduction of advantageous RNA modifications that cannot be generated biologically.

**Specificity**

Systematic comparison of a broad spectrum of synthetic ASOs with modified backbones (Figure 4) revealed that high-affinity modifications, for example, the incorporation of locked nucleic acid, 2'-fluoro (2-F), or 2'-O-methyl bases, increase potency but maintain specificity. 2'-O-methyl/locked nucleic acid chimeric ASOs with phosphorothioate linkages exchanging a nonbridging oxygen of a phosphodiester bond with sulfur (conferring nuclease resistance at this site) were the most

---

**Table. Future Directions in Cardiovascular RNA Interference**

<table>
<thead>
<tr>
<th>Investigation</th>
<th>Novel Strategies</th>
<th>Future Perspectives</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiac-targeted RNAi</td>
<td>Cardiotropic AAV9-based silencing of Ca2+ cycle regulator phospholamban</td>
<td>Cardiac-specific gene knockout for therapeutic purposes, and as an alternative for cardiac-specific inducible knockout models</td>
<td>3</td>
</tr>
<tr>
<td>Monocyte-targeted RNAi</td>
<td>Nanoparticle-encapsulated synthetic siRNA for silencing of monocyte chemokine receptor CCR2 in myocardial infarction</td>
<td>Nonviral RNA delivery system targeting monocytes in vivo, with high translational potential</td>
<td>4</td>
</tr>
<tr>
<td>RNAI imaging in vivo</td>
<td>PHD2-shRNA sequence followed by a hypoxia response element-containing promoter driving a firefly luciferase reporter gene</td>
<td>Imaging of RNAi distribution in space and time in experimental animal models</td>
<td>59</td>
</tr>
<tr>
<td>Functional improvement of stem cells by RNAi</td>
<td>To ameliorate a limitation of cardiovascular stem cell therapy, RNAi was used to enhance survival and function of transplanted cells</td>
<td>Possible enhancement of the efficacy of cardiovascular stem cell therapies</td>
<td>60</td>
</tr>
<tr>
<td>Allele-specific RNAi</td>
<td>Allele-specific RNAi for treatment of human-induced pluripotency stem cell cardiomyocytes</td>
<td>Rescue of autosomal-dominant-negative disorders eg, long-QT syndrome</td>
<td>61</td>
</tr>
<tr>
<td>Induction of alloimmune tolerance by RNAi</td>
<td>In heart transplantation models, RNAi induced alloimmune tolerance through silencing of TLR adaptors MyD88 and TRIF</td>
<td>In vivo treatment of recipients with siRNAs to MyD88 and TRIF prolonged allograft survival.</td>
<td>62</td>
</tr>
<tr>
<td>Plaque stabilization by RNAi</td>
<td>Lentivirus-based RNAi to silence chymase increased plaque stability in atherosclerosis in vivo</td>
<td>Chymase as a target for plaque stabilization in atherosclerosis, vasculature as an RNAI target</td>
<td>70</td>
</tr>
</tbody>
</table>

AAV indicates adeno-associated virus; CCR2, C-C chemokine receptor type 2; PHD, prolyl hydroxylase domain protein 2; shRNA, short hairpin RNA; siRNA, short interfering RNA; TLR, toll-like receptor; and TRIF, TIR-domain-containing adapter-inducing interferon-β.
potent constructs tested for miR inhibition in vitro. Such in vitro studies are of course only 1, but an important, step toward development of in vivo therapies because whatever synthetic targeting system is used it will need to encompass such modifications. As long as nonviral ncRNA therapy is attempted, these findings may well direct the choice of the therapeutic RNA type. Unfortunately, combination of these high specificity modifications with vector systems is impossible. Of current analytic and possible future therapeutic interest is the discovery that seed-targeting tiny locked nucleic acids may be deliberately used to elucidating the functions of entire miR families in vivo, as opposed to the commonly attempted modulation of single miRs.

**Stability**

RNAi may be triggered by introducing chemically synthesized siRNAs into a cell system. A more stable knockdown, however, requires the use of plasmids or viral vectors encoding shRNAs as precursors of siRNA. This requirement likewise applies to miR modulation therapies, which may have complex consequences in the recipient dependent on biochemical stability and distribution of the therapeutic RNA. The challenge to produce siRNAs in vivo continuously was met by designing vectors expressing shRNAs or premiRs from RNA polymerase III promoters (U6, H1, 7SK), which are processed into active siRNAs or miRs in target cells. On the contrary, miR inhibition can be achieved by chemically synthesized Antagomirs (cholesteryl-conjugated 2′-O-methyl ASOs), peptide nucleic acids, or backbone-modified small RNAs to improve biostability, target miRNA affinity, or cellular uptake (Figure 4), or by vector-based recombinant expression of miR Sponges or Erasers which act by miR scavenging, or Target Masks blocking miR binding. Antagomirs seem to exert rather long-lived effects and may for certain applications circumvent the need for viral vectors.

**Targeting**

The originally developed AAV vector prototypes have been derived from AAV serotype 2 (AAV2). AAV2 vectors had low transduction efficiencies in vivo and displayed no cardio tropism. This problem has been overcome. First, because vector genomes with intact AAV2-inverted terminal repeat can be packaged into the capsids of other AAV serotypes by pseudo typing. Second, the identification of a large number of new AAV serotypes with diverse properties allowed the development of AAV vectors with tropism for different organs in vivo. In the first pioneering clinical studies, an AAV1 vector has been successfully used for the treatment of severe heart failure by cardiac overexpression of sarcoplasmic reticulum...
Ca²⁺-ATPase 2a. Availability of multiple AAV serotypes for cardiac transduction is highly desirable because some patients may have preexisting antibodies against certain AAV serotypes, or develop them after AAV vector application. In these cases, switching to another serotype would allow continuation of therapy. Importantly, targeting of therapeutic genes or regulatory RNAs to an organ of choice may be achieved in some cases by simple intravenous vector injection. AAV9 pseudotyped vectors are highly efficient for cardiac-targeted gene or RNAi transfer in mice and rats, with ≈80% cardiomyocyte transduction after intravenous application. In rodents, AAV9 showed low expression in other organs. Cardiac specificity is further improved by liver detargeting by incorporation of target sites for liver-specific miRs in the 3′-untranslated region of the transgene or shRNA or miR expression cassette. Liver detargeting shall avoid oversaturation of cellular miR/shRNA pathways which may result in liver toxicity. Capsid-modified AAVs have been generated by DNA shuffling of different AAV serotype capsid genes to improve targeting properties. Because of species-differences it not yet entirely clear which AAV serotype is best suitable for ncRNA therapy in humans, although AAV9 has already been shown highly efficient in nonhuman primates.

Transcriptional targeting is useful to further confine synthesis of ncRNAs to the target. Improved cardiac specificity was achieved using modified myosin-light-chain promoters for AAV vector-mediated gene therapy, but these polymerase II promoters are unsuitable to express conventional shRNAs which are efficiently transcribed by polymerase III (U6, H1, 7SK) promoters only. Importantly, naturally occurring miR sequence may be replaced by an artificial shRNA sequence embedded into the original miR environment, to achieve classical transcriptional targeting of the therapeutic shRNA sequence to an organ of interest. This discovery has enabled expression of shRNAs from tissue-specific promoters, as previously used in classical gene therapy. Recently, post-transcriptional targeting was introduced which is based on miR target site incorporation into the expression cassette. Thus, a genetically engineered miR-155–based shRNA has been shown to have similar silencing efficacy but less immunogenecity than a previously successfully used AAV9-RNAi vector targeting the cardiac regulator protein phospholamban (H. Fechner et al, unpublished, 2013). Multiplex microRNA strategies with several miR-based shRNAs expressed from a common polycistronic miR structure have also been used, and may become ic miR structure have also been used, and may become useful for the treatment of viral infections, where cosilencing of multiple virus-encoded genes significantly improves antiviral efficacy.

 Whereas biological vectors are characterized by complex and largely preformed targeting properties (Figure 4), synthetic RNAi delivery systems must be build up step by step. If both the therapeutic RNA and delivery vehicle, for example, nanoparticle, are sufficiently stable in vivo (blood plasma, cytoplasm), their traffic in vivo can be modulated. Thus, in situ siRNA–albumin conjugation enhances extravasation, stability, and cardiac gene silencing in vivo. A Gal-PEG-Et nanoparticle carrying angiotensinogen shRNA nanoparticle shRNA transfection system mediated RNAi in early atherosclerotic lesions. Aptamers and other ligands were used to address receptors on target cells. Importantly, however, such ligand–receptor interactions are only efficient in vivo if the delivery vehicle is able to cross endothelial barriers. A strictly local delivery system of high interest to cardiovascular medicine are siRNA-eluting surfaces using technologies related to drug-eluting stents. Because of the high technological standard in this field, further investigation of opportunities for RNAi delivery is warranted.

Side Effects
Several potential application problems and side effects of both synthetic therapeutic small ncRNAs and vector-based ncRNA delivery systems arise from immune system stimulation. First, AAV vectors cannot be used in patients with preexisting antibodies or lose their efficacy on development of such antibodies in treated patients. Second, even subtle structural differences between synthetic or recombinant small RNA molecules and endogenous counterparts, for example, miRs, may activate the innate immune system through both toll-like receptor-dependent and toll-like receptor-independent mechanisms, and considerable efforts have been made to define structural requirements to avoid this problem. Only in noncardiovascular diseases, for example, cancer, immunostimulation by ncRNAs may be an acceptable or even useful side effect. Third, RNAi triggers, for example, shRNAs, may critically compete with endogenous miR biosynthetic pathways leading to severe toxicity. Here again detailed understanding of specific sequence determinants may help to avoid toxicity.

Regulatability
Major efforts have been undertaken to make vector-based gene and also ncRNA delivery system regulatable, with the prospect to adjust their levels to possible changing requirements. A desirable feature of ncRNA vectors would also be complete switch-off function. Several systems of possible clinical relevance have been reviewed elsewhere.

Clinical Translation of Findings From Proof-of-Principle Studies
First, given the enormous amount of work and money required to develop ncRNA therapeutics, it may be advisable to focus efforts on targets of potential interest in large patient cohorts. Second, targets should be disregulated in human cardiovascular diseases as documented by patient-derived data. Third, their relevance should be documented by proof-of-principle studies in animals. Unfortunately, for several important human cardiovascular diseases no adequate animal models exist. Fourth, there should be assessment of the chance for development of conventional small molecule drugs mimicking the desired effect of the ncRNA sequence in vivo. In many instances, however, the development of receptor or pathway blocking agents was unsuccessful (eg, for phospholamban). Fifth, there should be a stable system available for ncRNA sequence delivery to the desired tissue. Such systems may be tailor-made synthetic small RNAs in isolation, coupled to proteins or nanoparticles, or AAV-based vectors targeting cardiovascular structures.
RNAi: Search Tool for Novel Therapeutic Targets in the Human Genome

Search Strategies to Identify ncRNAs of Likely Clinical Relevance

Against the background of the complexities on the noncoding genome, one may adopt a pragmatic heuristic approach (Figure 5) directed at the identification of noncoding therapeutic targets in human cardiovascular diseases. Data and insights from genome-wide phylogenetic studies, comprehensive sequencing work, and animal models of human diseases constitute an indispensable first step toward target identification. However, the functions of the same ncRNA may differ between species, and most animal models differ significantly from the respective human disorder. Moreover, some ncRNAs do not exist in humans, such as the lncRNA Braveheart, or have no close homologues (several miRs), so that their functions and possible pathogenic relevance cannot be adequately investigated in animal models. It, therefore, seems suitable and necessary to conduct transcriptome mapping also directly in humans, and in the specific diseases of interest. Because of the advancement of technologies, comprehensive transcriptional mapping is possible even from the minute quantities of RNA obtained from cardiac or other human biopsy samples. It is entirely possible that therapeutic proof-of-concept studies addressing ncRNAs deregulated in animal models cannot be efficient in humans because of such interspecies differences.

Functional Spectrum and Therapeutic Potential of Classical RNAi

The mechanism of RNAi is conserved across species and involves small RNAs inhibiting translation or degrading mRNA by PTGS, which may protect against viral infection and regulate endogenous genes. Several RNA classes exert PTGS: siRNAs with perfect complementarity to targets cause transcript degradation; miRs with imperfect complementarity cause translational repression; and piRNAs target transposon transcripts in animal germ lines. siRNA-mediated RNAi is an efficient broad spectrum antiviral mechanism, whereas immunologic systems are rather virus specific. RNAi has potential for the treatment of viral cardiomyopathies in conjunction with antiviral drugs. Interestingly, several viruses generate small RNAs capable to modulate host gene expression to their advantage. miR evolution has reached 63 families in mammals and 1424 separate miR genes in humans (Figure 1). Genome-wide assessment of miR targets suggests that in humans ≈50% of miRNAs are influenced by miR regulation. Regarding the functional assignment of miRs in the cardiovascular system, an interesting approach is high-throughput functional screening in cell cultures using a whole-genome miR library. Using this screening and further selection steps, a recent investigation has identified miRs promoting cell cycle reentry of adult cardiomyocytes. After MI, these miRs stimulated cardiac regeneration and recovery of cardiac functional parameters in mice. For the cardiovascular system, several recent reviews have discussed the differential regulation of miRs, their functions during development, roles in disease pathogenesis, and therapeutic perspectives, and these small ncRNAs are, therefore, not covered here. One difference between RNAi and miR-modulating therapies should be emphasized, however, that is, deliberate modulation of 1 single target by RNAi, whereas miR modulation influences multiple targets as integral part of miR action. Endogenous miR genes are also subject to regulation via their promoter, similar to protein-encoding genes, and miR modulators antagonists or mimics overdrive this physiological regulatory network with possible implications for the therapeutic setting. Complexity is added to the endogenous miR systems by extracellular miR trafficking via vesicles, the existence of genetic miR variants, and strong disease-context dependency of miR actions. Despite several similarities between RNAi and miR systems, their functional biology is grossly distinct.

Functional Assignment and Therapeutic Perspective of lncRNAs

After the discovery of RNAi, miRs, and other small regulatory RNAs had already revolutionized our perception of gene regulation in higher organisms, a huge number of other cellular players was added when a major fraction of the transcriptome...
was found to encompass not small RNAs of the known types, but far larger lncRNA transcripts expressed in a cell type and development stage-specific manner. This closely regulated manner of expression, in conjunction with biochemical, mechanistic, and functional findings for an as yet small number of paradigmatic lncRNAs, indicates high importance of this novel class of ncRNA in cardiovascular health and disease. Clinical interest was triggered by their involvement in viral infectious diseases, immune disorders, and stem cell differentiation and function. Regarding cardiovascular disorders, recent studies suggest involvement of a number of lncRNAs in cardiac development and important cardiac diseases. As excellent recent reviews have addressed this topic, we focus here on the discovery path of the lncRNA data currently available, issues that need to be further investigated by inclusion of cardiac transcriptome data from patients, and first evidence suggesting therapeutic potential of lncRNA systems.

Assignment by Genome-Wide Association Studies
A first type of data stems from genome-wide association studies analyses. Human chromosome 9p21.3 region is a strong genetic susceptibility locus for coronary artery disease, but no association with traditional risk factors, common protein-coding variation, variants in splice donor/acceptor sites, or copy number was observed. However, strong association with an isoform of lncRNA ANRIL and cyclin-dependent kinase inhibitor 2B was found and led to the hypothesis that complex regulatory variation affecting ANRIL and cyclin-dependent kinase inhibitor 2B gene expression explains the increased MI risk linked to this locus. ANRIL modulates attherosgenic pathway expression in vascular smooth muscle cells in vitro, different ANRIL isoforms had differential effects, and ANRIL was induced by the ataxia telangiectasia mutated kinase-E2F1 transcription factor signaling pathway. Another lncRNA of cardiovascular relevance identified by genome-wide association studies analysis is MIAT/Gomafu. Single nucleotide polymorphisms in this locus in complete linkage disequilibrium were associated with MI, but the underlying mechanism is unknown to date.

Assignment by Experimental Targeted Search
Braveheart, an lncRNA required for cardiovascular lineage commitment, was identified by a systematic targeted search for lncRNAs restricted to specific cell types during embryonic stem cell differentiation. lncRNA expression profiling by RNA-seq in mouse embryonic stem cells and differentiated tissues led to identification of a number of candidates which were then individually further evaluated. Braveheart interacts with a component of polycomb-repressive complex 2 during cardiomyocyte differentiation and is thought to regulate cardiac commitment epigenetically. One remarkable aspect of this study, with regard to the identification of lncRNAs of potential clinical relevance, is the observation that the murine Braveheart has obviously no human homologue. The interesting question arises whether this lncRNA locus has been lost during human evolution, with a resulting loss of certain capabilities on cardiac biology, or whether it was substituted in humans by a structurally distinct (and thus not recognizable by traditional genome sequence comparison) transcriptional system.

Assignment by Transcriptome Mapping in Patients
This discovery path led to cardiovascular interest in the evolutionary highly conserved lncRNAs MALAT1 and NEAT1. MALAT1 and NEAT1 are located <70 kb apart on human chromosome 11. MALAT1 is post-transcriptionally processed by a novel biosynthetic pathway involving RNAses P and Z, to yield a small tRNA-like product designated MALAT1-associated small cytoplasmic RNA (mascRNA; Online Figure I). To identify functions of the MALAT1 system, murine knockout models have been used which showed that loss is compatible with normal development and life under normal laboratory conditions, and that its function becomes apparent only in specific cell types and under particular conditions. A pioneering recent study revealed that MALAT1 is a critical regulator of the metastasis phenotype of lung cancer cells. By endomyocardial biopsy-based cardiac transcriptome mapping, we recently found MALAT1 dysregulated in human cardiomyopathies. Modulation of the small lncRNA-product mascRNA by recombinant overexpression or PTGS strongly influenced intrinsic cellular immunity and may have therapeutic potential (Poller et al, unpublished data, 2013). Cardiac transcriptome maps of lncRNAs here yielded first evidence of their involvement in cardiac diseases, exemplifying the search strategy for lncRNA functions of clinical interest as outlined in Figure 5.

Processing of lncRNAs
It will be preferable to use RNA-seq instead of microarray technology to achieve a comprehensive overview, including not only the primary transcripts, but also their specific processing products, which are likely to be carriers of defined cellular functions. Different transcripts (MEN β, MEN ε) also originate from the NEAT1 locus, and the nonpolyadenylated 3‘-end of MEN β is noncanonically processed by RNases and yield a tRNA-like product designated as menRNA (Online Figure II). This complex processing seems to be integral part of the NEAT1 system. Live-cell imaging system allowing inducible transcription of NEAT1 revealed that it serves as a platform to recruit proteins to assemble paraspeckles, which are assumed to control several biological processes, including stress responses and cellular differentiation. It was recently shown that NEAT1 influences virus infections. Regarding the general aspect of differential cellular functions of lncRNA processing products it is of interest that another NEAT1-related sequence designated as trophoblast ncRNA inhibits alloresponses by suppressing class II transactivator promoter III and major histocompatibility complex class II expression in murine B-lymphocytes. An important genome-wide analysis of lncRNA stability has determined the half-lives of ~800 lncRNAs and ~12 000 mRNAs, as a further step toward kinetic characterization of lncRNA systems.

RNAi: Toward Therapeutic Exploration of lncRNA Systems
RNAi for the Functional Assignment of lncRNA Systems
RNAi recently emerged as an important novel tool to identify the physiological cardiovascular functions, and hence possible
therapeutic potential, of newly identified IncRNAs.\textsuperscript{143} RNAi-based functional profiling was applied to 56 loci found by genome-wide association studies to be associated with coronary artery disease and MI. RNAi was used to analyze 133 candidate genes in these genomic regions showed that individual genome-wide association studies loci may contain >1 gene with cholesterol-regulatory functions. Several genes without previously known lipid-regulatory roles were functionally assigned using silencing by RNAi. This study demonstrates that quantitative, cell-based RNAi is an important strategy for identification of possible novel therapeutic targets.

**RNAi for the Therapeutic Modulation of IncRNA Systems**

Several of the basic principles and methods with clinical translational potential as discussed in the previous article on RNAi are also applicable to IncRNA modulation. Thus, it is possible to block even very long precursor transcripts by targeting different regions of it with a panel of ASOs, independent of methods such as homologous recombination or zinc finger nucleases.\textsuperscript{130} To date, however, limitations arise from our incomplete knowledge of IncRNA processing and kinetics. Furthermore, primary IncRNA transcripts generally seem to have such a large number of nucleic acid and protein-type interaction partners, and highly complex functions that adequate control of possible side effects of IncRNA blockade seems impossible to achieve. It should be kept in mind, however, that blockade or overexpression of an entire IncRNA would be analogous to blockade or overexpression of a primary miR which is not the principle of miR therapeutics. To the contrary, only mature miRs as highly specific processing products were ever considered for clinical application, and this would be analogous to the targeting of single processing products of a IncRNA of interest (eg, mascRNA or menRNA; Online Figures I and II). From a therapeutic perspective, research into the functions of IncRNAs in the cardiovascular system, several of which apparently have impact in cardiovascular pathogenesis, should pay attention to these aspects in both experimental studies and transcriptome analyses of cardiovascular patients.

**Concluding Remarks—Therapeutic Exploration of the Human Genome**

Because of the now apparent extreme complexity of the human genome, one may consider a biological analogy to Heisenberg Uncertainty Principle in physics: Intact biological systems encompassing all molecular components, including the plethora of newly discovered ncRNAs, can be dissected into small subsystems accessible to reductionistic molecular characterization. By doing so, however, the original biological context and functions are lost because in the intact living organism the subsystem studied is in fact intricately linked to multiple neglected but essential parts. This might be a fundamental limitation, to be only partially overcome by combining data from different study systems, including thorough genomic and transcriptomic investigations in patients using state-of-the-art tools.

Apart from this fundamental limitation, it is currently obviously impossible to simulate the behavior of a normal or diseased cell without knowledge of all relevant players. Until 2 decades ago, one might have hoped that this could change in the foreseeable future, with a comprehensive data set on the then known players. Even that would have been a tremendous endeavor, followed under the headlines of Systems Biology and Network Medicine,\textsuperscript{144} but with the recent discovery of a huge number of previously unknown and even unsuspected players from the noncoding human genome\textsuperscript{145–149} this goal has certainly shifted far away. It is already entirely clear that it will take a very long time, if ever possible, to arrive at a full understanding of the workings of the human genome with its multiple and exceedingly complex layers of regulation and dynamic adaptation to changing requirements.

Whereas comprehensive understanding of the workings of the genome is beyond reach now, current knowledge will nevertheless become increasingly valuable for cardiovascular research and medicine. If the complementary insights from evolutionary biology, genomic and transcriptomic studies in well-selected cardiovascular patient cohorts, and experimental evaluation of possible ncRNA therapeutic candidates in cell and animal models are brought together; currently available technologies are likely to reveal a continuously growing panel of novel targets for cardiovascular therapy. From a pragmatic perspective, a broad spectrum of most valuable discovery tools for target prospecting in the noncoding genome is already at hand, as well as various delivery tools suitable for clinical translation. Because of the complexity of the task, clinical translation in this area continues to be a most interesting and suitable area for close interdisciplinary research.

**Sources of Funding**

This work has been supported by Deutsche Forschungsgemeinschaft through Collaborative Research Center Transregio 19 by grants W. Poller and C. Skurk. The other authors report no conflicts.

**Disclosures**

None.

**References**


Identification of a novel non-coding RNA, MIAT, that confers risk of myocardial infarction.


myocardial infarction.


Cardiovascular RNA Interference Therapy: The Broadening Tool and Target Spectrum

**Supplemental Figures I and II**

**Primary Transcription and Processing of the Long Noncoding RNAs MALAT1 and NEAT1**

Referring to Fig. 5 which outlines therapeutic perspectives of lncRNAs and their processing products, the graphs below show details regarding the long primary transcripts from the MALAT1 (Figure I) and NEAT1 (Figure II) genomic locus and their peculiar posttranscriptional processing by RNAses.

**Figure I:** Whereas the large \(\approx7\) kb primary MALAT1 transcript sequence is overall not well conserved across species (sequence comparison, Panel A), a 61-nt tRNA-like processing product generated by RNAase P and RNAse Z and designated as mascRNA, is highly conserved (Panel B)\(^{37,133}\). MALAT1 was recently observed to be deregulated in human viral cardiomyopathies, and recombinant mascRNA modulates the antiviral immune response (Poller et al., unpublished), suggesting possible pathogenic relevance of the MALAT1 system. From a therapeutic perspective, it is of interest that these findings were triggered by systematic cardiac transcriptome mapping of cardiomyopathy patients, whereas this system had not been linked to cardiac diseases before. This example also illustrate difficulties which may arise in searches for lncRNAs of therapeutic interest. Sequence conservation is low when the whole primary transcripts are considered, and without further information as available for MALAT1 and NEAT1 it is nearly impossible to identify any domains of particular functional importance. Further, measurement of both precursor and products expression in diseased organs is required to obtain a complete picture of the lncRNA system.

**Figure II:** Different primary transcripts (\(\approx23\) kb MEN\(\beta\), \(\approx3.7\) kb MEN\(\gamma\)) arise from the NEAT1 locus near MALAT1 on human chromosome 11q13.1. As MALAT1, NEAT1 is also overall not well conserved across species (Panel A). Similar to MALAT1, MEN\(\beta\) is also processed by RNAase P and RNAse Z to yield a small tRNA-like product designated as menRNA (Panel B)\(^{38,39,47,136,142}\). From the biochemical view it is remarkable that mascRNA and menRNA are generated by similar biosynthetic pathways, and most probably these small RNA products have functions distinct from their large precursors, and different intracellular kinetics and trafficking. Whereas MALAT1 is intensively investigated in oncology, however, there is only fragmentary knowledge regarding MALAT1 and NEAT1 in the cardiovascular system despite evidence that they are deregulated in human cardiac diseases. As outlined in Fig. 4, the sequential steps of genomic discovery, structural characterization, functional genomic knockout of the whole lncRNA systems, and transcriptome mapping in various diseases, further tasks are the blockade or overexpression not only of the whole system, but also of single processing products, both in various cardiovascular cell types and animal models, using ablative or ncRNA transfer technologies as discussed above. Reference numbers in this legend refer to the main manuscript.
SUPPLEMENTAL FIGURE I

MALAT1 – masRNA Processing

A  ~7 kb primary transcript

1. RNase P

2. RNase Z

B  ~ 7 kb hMALAT1

triple helical structure at 3' end protects transcript from exonuclease degradation and thus confers stability

+ CCA
SUPPLEMENTAL FIGURE II

A  NEAT1 – menRNA Processing

= 23 kb primary transcript

50 bp processing product

U-rich motif  conserved stem loop  U-rich motif  A-rich tract

1. RNase P  2. RNase Z

B

~ 23 kb hMENbeta

triple helical structure at 3' end protects transcript from exonuclease degradation and thus confers stability