CHAPTER 7

Summary and General Discussion
State of the art of heart failure

Heart failure (HF) is a deadly and costly disease affecting 5.7 million people in the US alone, and a leading cause of hospitalization for those >65 years of age.\textsuperscript{1} It is the leading cause of death in industrialized nations and represents an enormous economic burden to society. Pathological hypertrophy of the heart muscle is a common process among numerous types of heart diseases, including ischemic diseases, hypertension and several genetic forms of cardiomyopathies.\textsuperscript{2} Therefore, sustained hypertrophic remodeling represents one of the major clinical predictors of heart failure in humans. Hypertrophy of the left ventricle is caused by chronic pressure overload (hypertension, aortic stenosis).\textsuperscript{3} This can lead to decreased cardiac output due to reduced systolic fractional shortening. As described in Chapter 1, distinct phenotypes of hypertrophy can be distinguished based on the geometry of the heart and individual cardiomyocytes. Concentric cardiac hypertrophy displays a reduced left ventricular chamber dimension and thickening of the left ventricular free wall and septum. Eccentric hypertrophy is characterized by ventricular wall dilatation and can occur after myocardial infarction. Concentric hypertrophy can progress to eccentric hypertrophy and dilation leading to systolic heart failure.

Current standard pharmacological therapy includes the use of inhibitors of angiotensin II, β-AR antagonists, aldosterone antagonists, and diuretics.\textsuperscript{4-6} Other modern therapies are interventional therapy (intracoronary stent implantation, balloon angioplasty, percutaneous valve repair),\textsuperscript{7} electrophysiological therapy (implantation of cardioverter defibrillator, cardiac resynchronization treatment, ablating arrhythmic foci),\textsuperscript{8} and surgical treatment (ventricular assist device implantation, heart transplantation).\textsuperscript{9} Current HF therapy is primarily focused on treating symptoms and fails to directly address the key underlying intracellular signal transduction abnormalities. Despite these different forms of treatments, HF-related mortality remains high. It should be noted that so far there are no pharmacological HF drugs that target intracellular targets, as those compounds are difficult to create due to biochemical challenges (cell permeability issues etc.). Furthermore, our understanding of the deranged signaling pathways inside the cell that lead to the progress of the disease is far from complete. A better understanding of molecular mechanisms of the change in cardiac remodeling is clearly needed.
The non-coding genome

The genome is the carrier of hereditary information that defines an organism. Results from the Encyclopedia of DNA Elements (ENCODE) project indicates that at least 80% of the human genome can be transcribed, yet only less than 2% of our genome encodes proteins.\textsuperscript{10} Accumulating evidence shows that there is a clear correlation between the functional complexity of a certain organism and the diversity of its non-coding genome. The main output of the genomes of complex organisms is genetically active but non-coding RNA (ncRNA).\textsuperscript{11} These ncRNAs act as integral components of the molecular networks in development and disease.\textsuperscript{12} Classification of ncRNAs is based on their length, function, biogenesis, polarity (sense or antisense), and protein-binding partners.\textsuperscript{13} According to their size, ncRNAs are subdivided into two groups: small ncRNAs (< 200 nt) and long ncRNAs. Long ncRNAs (>200 nucleotides) are relatively stable and do not undergo major processing before carrying out their normal functions.\textsuperscript{14} On the other hand, short ncRNAs (<200 nucleotides) are processed from longer precursors.\textsuperscript{15}

One of the most studied short ncRNAs are microRNAs (miRs), single-stranded small RNA molecules consisting of 20-23 nucleotides that are generated from endogenous hairpin transcripts.\textsuperscript{16} Numerous studies have identified alterations in expression levels of ncRNAs as a feature of heart failure, especially microRNAs (miRs). Therefore, considerable attention is focused on chemically targeting miR expression as a therapeutic solution for HF. In general, there are two approaches to developing miR-based therapeutics: miR antagonists and miR mimics. A new mechanism of action, the ability to function as master regulators of entire pathways and an apparent lack of adverse events in normal tissue make miR antisense inhibitors and miR mimics promising technologies for current and future therapeutics development. Ways to inhibit miR expression by antisense oligonucleotides are outlined in Chapter 3. For example, Regulus’ drug RG-101 is a GalNAc-conjugated anti-miR targeting miR-122 for the treatment of Hepatitis C virus (HCV). Their Phase I study is successfully completed, and the first Phase II clinical data are expected to be reported by the end of 2016 (http://www.regulusrx.com). Santaris Pharma, based in Copenhagen, Denmark has miravirsen (another antisense microRNA-122 drug), in Phase II clinical trials.\textsuperscript{17,18} Phase I studies confirmed preclinical findings results in rodents and nonhuman primates,\textsuperscript{17,19} which showed good tolerability of the drug. A complete overview of preclinical studies applying anti-microRNA oligonucleotides to inhibit specific miRs in relevant models of HF is provided in Chapter 3, Table 1 (page 57).
Besides inhibiting miR expression, it is possible to induce miR expression using adeno-associated virus (AAV) vectors. Although AAV9 is not widely used for miR overexpression yet, in various cases it has proven to be beneficial to stimulate expression of a particular miR, as also described for miR-148a by AAV9 in Chapter 4. Intraventricular delivery of AAV vectors induced long-term (18 months) overexpression of miR-669a and improved survival of β-sarcoglycan (Sgcb)-null mice, a model for muscular dystrophy. Treated dystrophic mice displayed reduced pathological remodeling, enhanced sarcomere organization and increased systolic fractional shortening of the left ventricle. Furthermore, AAV-mediated overexpression of hsa-miR-590 and hsa-miR-199a in mice, after myocardial infarction, reduced infarct size and significantly improved cardiac function because they trigger cardiomyocyte proliferation and cardiac regeneration after myocardial infarction. Other reports indicate that AAV–miR-378 attenuates cardiac hypertrophy after pressure overload because of thoracic aortic constriction. In rats, AAV-mediated overexpression of miR-1 lead to a marked reduction of myocardial fibrosis, improved calcium handling, inhibition of apoptosis, and inactivation of the mitogen-activated protein kinase signaling pathways, indicating prevention of maladaptive ventricular remodeling. AAV-mediated miR-overexpression studies in relevant heart failure models are summarized in Table 1.

Although there are no clinical trials yet for AAV-mediated overexpression of miRs, companies are developing miR mimics for therapy. Mirna Therapeutics developed a miR-34 mimic, called MRX34. MRX34 is a double-stranded RNA mimic of the tumor suppressor miR-34, which is delivered by liposome to inhibit multiple oncogenic pathways as well as to stimulate anti-tumor immune response to induce cancer cell death (www.mirnarx.com). MRX34 is currently in a Phase I clinical trial in patients with unresectable primary liver cancer, and advanced or metastatic cancer with liver involvement (ClinicalTrials.gov identifier: NCT01829971). If MRX34 proves to be promising, others will soon follow.
Table 1 Overview of preclinical studies using AAV to overexpress certain miRs in relevant models of heart failure

<table>
<thead>
<tr>
<th>miR</th>
<th>Dose/Delivery</th>
<th>Model</th>
<th>Target</th>
<th>Outcome</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AAV9, single-bolus $5 \times 10^{11}$ vg (viral genomes) tail-vein injection</td>
<td>Rat, pressure overload</td>
<td>Fibulin-2 (Fbln2)</td>
<td>Reversed cardiac hypertrophy, attenuated pathological remodeling</td>
<td>23</td>
</tr>
<tr>
<td>148a</td>
<td>AAV9, single-bolus $1 \times 10^{12}$ viral genomes, tail-vein injection</td>
<td>Mouse, TAC</td>
<td>Gp130</td>
<td>Improved systolic function after TAC, preventing transition of concentric hypertrophy towards dilation</td>
<td>Ch 4</td>
</tr>
<tr>
<td>199a</td>
<td>AAV9, single-bolus $1 \times 10^{11}$ vg intraperitoneally or intracardiac injection</td>
<td>Mouse, MI</td>
<td>Homer1, Hopx, Clic5</td>
<td>Marked cardiac regeneration and almost complete recovery of cardiac functional parameters after MI</td>
<td>21</td>
</tr>
<tr>
<td>378</td>
<td>AAV9, $1 \times 10^{12}$ genome copies, tail vein injection</td>
<td>Mouse, TAC</td>
<td>MAPK1, GRB2, KSR1, IGF1R</td>
<td>Attenuated thoracic aortic constriction–induced cardiac hypertrophy, improved cardiac function</td>
<td>22</td>
</tr>
<tr>
<td>669a</td>
<td>AAV2/9, $1 \times 10^9$ genome copies, intraventricular puncture in neonates</td>
<td>Mouse, DCM (Sgcb-null)</td>
<td>MyoD, Agtr1a, Il6, Il1b (not confirmed)</td>
<td>Reduced adverse remodeling, enhanced systolic fractional shortening of left ventricle in treated dystrophic mice</td>
<td>20</td>
</tr>
</tbody>
</table>

miR-148a as a regulator of cardiac hypertrophy

In Chapter 4 we showed that miR-148a is differentially and dynamically expressed in distinct subtypes of human and mouse forms of heart failure. Concentric hypertrophy in both human and mouse hearts correlates with increased miR-148a expression, whereas dilated cardiomyopathy is accompanied by a profound decrease in miR-148a myocardial expression levels. In the heart, miR-148a directly targets glycoprotein 130 (gp130). Gp130 is a shared receptor utilized by several related cytokines, including IL-6, IL-11, IL-27, Leukemia Inhibitory Factor (LIF), Oncostatin M (OSM), and Cardiotrophin 1 (CT-1). CT-1 is a member of the IL-6 family, and CT-1-mediated gp130 activation is well known to induce eccentric hypertrophic remodeling in cardiac myocytes. In vitro experiments demonstrated
that overexpression of miR-148a, by delivery of precursor molecules, inhibits eccentric cardiomyocyte hypertrophy after CT-1 stimulation in neonatal rat cardiomyocytes. In line, silencing of endogenous miR-148a results in spontaneous eccentric cardiomyocyte hypertrophy. In vivo inhibition of miR-148a, by using a specific antagonir, caused marked impairment in cardiac function and increased fibrosis, which was associated with an increase in gp130 expression. In addition, AAV9-mediated overexpression of miR-148a provoked a resistance to pressure-overload induced cardiac contractile dysfunction and dilatation.

In recent years, many efforts have been done to unravel the different mechanisms between concentric and eccentric cardiac remodeling. How molecular dysfunction evokes different patterns of cardiac remodeling is unclear. Our results demonstrate that miR-148a can prevent the transition from concentric hypertrophy induced by pressure-overload towards eccentric hypertrophic remodeling and dilation of the left ventricle. This indicates that the different forms of hypertrophic remodeling are induced by distinct molecular programs.

A recent study from the Molkentin lab describes a tension-based model, the force-time integral of cardiomyocyte function, that distinguishes between hypertrophic versus dilated cardiomyopathy. They used an array of genetically altered mice that permit the systemic tuning of sarcomeric tension generation to assess consequences on cardiac remodeling and disease. This study focused on hereditary conditions affecting cardiac sarcomeric proteins. Well-characterized functionally opposite mutations of the calcium-sensing thin filament sarcomeric protein, cardiac troponin C (cTnC) were used that quantitatively shift calcium-binding and tension-producing features of the myofilaments at a fixed amount on either side of normal to correlate with hypertrophic remodeling of the heart. The computational model of the integral of myofilament tension development predicted HCM and DCM in mice associated with essentially any sarcomeric gene mutations. They were also able to accurately predict human cardiac phenotypes from data generated in induced-pluripotent-stem-cell-derived myocytes from familial cardiomyopathy patients. Whether miR-148a is directly activated by changes in tension of the heart muscle has yet to be determined.
Phenotypic screening to identify microRNAs involved in CT-1-mediated cardiac hypertrophy

CT-1 is known for many years for its ability to induce eccentric hypertrophy in neonatal cardiomyocytes. However, few articles report whether chronic CT-1 administration leads to eccentric cardiac remodeling and altered cardiac function in vivo. López-Andrés and colleagues showed that CT-1 treatment increased left ventricular volumes, reduced fractional shortening and ejection fraction, and induced myocardial dilatation and myocardial fibrosis in Wistar rats treated with 20 μg/kg per day during 6 weeks.26 To the best of our knowledge, our study is the first demonstration that chronic CT-1 administration leads specifically to dilatation without an initial concentric hypertrophic response in mice. The finding that microRNA miR-148a directly targets Gp130, a key component of this CT-1 pathway, identifies a possible additional site of therapeutic intervention for heart failure by the class of RNA therapeutics.

Since we found that a specific miR can control the CT-1 pathway, we wanted to know if there are additional miRs that exert a similar effect. To this end, we performed a phenotypic high-throughput screen in neonatal rat cardiomyocytes stimulated with CT-1. CT-1 is a 201 amino acid protein member of the IL-6 family, that signals through a unique receptor system, consisting of leukemia inhibitory factor receptor beta (LIFRb) and a common signal transducer, the glycoprotein 130 (gp130).27 Signal transduction via gp130 involves at least three major downstream pathways: the janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, the Ras-Raf mitogen-activated protein kinase (MAPK, MEK/ERK) signaling cascade, and the phosphatidylinositol 3-OH kinase (PI3K)/Akt pathway.27 Multiple studies have shown that CT-1 plays a dual role in the biophysiology of the myocardium, providing protective effects on the one hand, but predisposing the heart to pathological conditions on the other hand. It was shown that CT-1 promotes neonatal cardiac myocyte survival and proliferation and is required for cardiac myocyte maturation.28 Treatment of cardiac cells with CT-1 induced heat shock protein (hsp) 70 and hsp90 synthesis and protected these cells against subsequent exposure to severe thermal or ischaemic stress.29 Another study showed that CT-1 can exert a protective effect against the damaging effects of simulated ischaemia/reoxygenation both when added after the simulated ischaemia at reoxygenation or when added prior to the simulated ischaemia. These protective effects were mediated by the p42/p44 MAPK pathway downstream of CT-1 activation.30
Aside from its protective effects, CT-1 activation is also implicated in pathological cardiac remodeling. CT-1 was originally isolated for its capacity to induce hypertrophy in neonatal cardiac myocytes, causing eccentric hypertrophy with an increase in cell length without a significant change in cell width. Sarcomeric units were assembled in series rather than in parallel as is observed in concentric hypertrophy. In humans, increased CT-1 serum levels have been observed in patients with hypertensive heart disease. CT1 is more elevated in hypertensive patients with left ventricular hypertrophy than in patients that have normal ventricular thickening. Cardiac CT-1 secretion is stimulated by ventricular stretch in perfused rat hearts. Also in patients with aortic stenosis plasma levels of proBNP and CT-1 were elevated and both N-terminal proBNP and CT-1 levels correlated to the maximum trans-valvular aortic pressure gradient. CT-1 was the most significant predictor of the severity of aortic stenosis. Furthermore, expression levels of CT-1 were significantly increased in the failing left ventricular myocardium of patients with end-stage heart failure compared with non-failing donor hearts. Plasma CT-1 levels are increased in patients with dilated cardiomyopathy (DCM) and significantly correlated with the LVmass index, indicating that CT-1 plays an important role in structural left ventricular remodeling in patients with DCM. Also after myocardial infarction, CT-1 plasma levels are elevated in humans and correlate with the degree of left ventricular systolic dysfunction.

CT-1 has multiple functions that can result in opposite outcomes. CT-1 can promote proliferation and survival of cardiomyocytes but also can cause cardiac hypertrophy and ventricular remodeling. Also, it is able to regulate several organ systems besides the cardiovascular system, and it activates different signaling pathways. These facts make it difficult to target CT-1 for therapeutic purposes. Directing CT-1 therapeutics to the target organ is a key challenge that requires further investigation. The different activities of CT-1 reflect the different signaling pathways that are activated by CT-1. Thus targeting a microRNA that is involved in one specific downstream pathway could tackle the dual activity issues of CT-1. Since expression of particular miRs is in most cases not organ-specific, delivery to the target organ is again key in developing a therapeutic strategy.

In Chapter 5, we used CT-1 as a hypertrophic stimulus on neonatal rat cardiomyocytes, and performed a high-content microscopy, high-throughput functional screen for miRs that influenced neonatal cardiomyocyte eccentric remodeling using a whole-genome miRNA library. The goal of this approach was to gain more insight in the signaling cascades and molecular mechanisms involved in
eccentric remodeling and dilatation. From our screen we identified miR-125b-5p, miR-542-3p, miR-151-5p and miR-532-3p as specific inducers of eccentric hypertrophy. None of those miRs has been studied in relation to eccentric hypertrophy and cardiovascular disease. Functional analysis of the gene involvement in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways revealed enrichment for genes belonging to ‘Pathways in cancer’, MAPK-signaling pathway’, ‘Endocytosis’, and ‘Regulation of actin cytoskeleton’ as top categories. Further experimental validation implicating these miRs in directly targeting genes in those pathways is still needed.

Other screens performed in cardiomyocytes have focused on identifying miRs in proliferation\(^2\), and miRs in concentric hypertrophy\(^3\). As expected, our top candidates were not among the candidate miRs in aforementioned 2 screens. An alternative approach, not including miRs, to differentiate hypertrophic signaling pathways was done by quantifying the contribution of individual pathways to specific changes in shape and transcript abundance. Cardiac myocytes were stimulated with 15 hypertrophic agonists and subsequently quantified for differential regulation of 5 shape features using high-throughput microscopy and transcript levels of 12 genes using qPCR. While hypertrophy pathways are highly connected, the agonist screen revealed distinct hypertrophy phenotypic signatures for the 15 receptor agonists they tested. They reported strong correlations between Bax and connective tissue growth factor (CTGF) mRNA abundance in response to angiotensin II and between myocyte elongation and CITED4 mRNA abundance in response to Nrg1. In their model, Nrg1 stimulates myocyte elongation and CITED4 expression, LIF stimulates myocyte elongation, and CITED4 expression negatively regulates myocyte elongation. Overexpression of CITED4 does not affect elongation of unstimulated cardiac myocytes. However, overexpression of CITED4 increased cell size and induces myocyte proliferation. Increased CITED4 may therefore be beneficial to the heart by preventing high levels of myocyte elongation.\(^3\) Although they also used CT-1 as one of the hypertrophic stimulants, no results on those experiments are described in their main report. In the supplemental data it is showed that CT-1 stimulation is followed by a significant increase in mRNA levels of IkB and VEGF, but in none of the other genes tested.
**Bigheart: a key IncRNA in the heart**

It has become increasingly evident that besides miRs, another class of ncRNA, IncRNAs, plays a key role in cardiac physiology and pathology. An overview of IncRNAs involved in control of gene regulatory programs in cardiac development and disease is provided in Chapter 2. In Chapter 6, we profiled IncRNAs in hearts suffering from non-ischemic, pathological hypertrophic remodeling using two well-established mouse models of heart failure: a surgical model of transverse aorta constriction (TAC) to induce sustained cardiac pressure overload and mice with heart-restricted calcineurin overexpression. We then selected IncRNA candidates that showed a similar differential expression pattern in both hypertrophy models and were not studied in this context before. We selected AK014683 (Ensembl ID 4833412C05Rik, or Bigheart) because it showed 5-15 fold differences with WT hearts. Bigheart was previously described to be upregulated in heart disease models, but its function was still unknown. We used RNA interference (RNAi) to silence the IncRNA Bigheart and demonstrate its functional requirement for agonist-induced cardiomyocyte hypertrophy. GO-analysis-based bioinformatics using the NPInter database that documents experimentally verified functional interactions between noncoding RNAs and biomolecules revealed that Bigheart is involved in mRNA processing. It was previously shown that Bigheart expression is increased in a cardiac-specific heterogeneous nuclear ribonucleoprotein U (hnRNP U) knockout model of cardiac disease. Loss of hnRNP U resulted in context-dependent splicing defects, since mixed splicing activities (skipping and inclusion) were observed. Indeed, our analysis using the NPInter database revealed that one of the verified interaction partners of Bigheart is MOV10, which is involved in pre-mRNA processing. Further validation to confirm that MOV10 is responsible for the Bigheart-mediated effect on cardiac hypertrophy is still required. Besides this new IncRNA Bigheart, our array also identified previously verified IncRNAs in heart failure. We detected Braveheart, a critical regulator of cardiac cell fate, and MALAT1, a regulator of endothelial cell function and vessel growth, confirming the accuracy of our results.

**Not all IncRNAs are IncRNAs**

Many non-coding RNA transcripts (including IncRNAs) contain multiple putative small open reading frames (ORFs) that can potentially be translated and have a coding function. There is increasing evidence that short ORFs present in some ncRNAs (including IncRNAs) are actually translated into small bioactive peptides, the
abundance of which is probably greatly underestimated. The polished rice (pri) or tarsal-less (tal) gene in Drosophila was originally annotated as a lncRNA, but demonstrated to encode a series of 11– to 32– amino acid peptides that orchestrate epidermal differentiation through the control of Shavenbaby transcriptional activity.\(^{43}\) Shavenbaby is a transcription factor and it was found that multiple genetic changes in the cis-regulatory region of shavenbaby caused the loss of dorsal cuticular hairs (quaternary trichomes) in larvae of Drosophila sechellia.\(^{44}\)

Recently, it was discovered that a skeletal muscle-specific lncRNA encodes a conserved micropeptide. Bioinformatic analysis revealed a short 138 nucleotide ORF with the potential to encode a highly conserved 46 amino acid micropeptide, named myoregulin (MLN).\(^{45}\) The MLN micropeptide is highly conserved across mammals and shows a strong structural resemblance to phospholamban (PLN) and sarcolipin (SLN), both of which directly interact with the sarco-endoplasmic reticulum Ca\(^{2+}\) adenosine triphosphatase (SERCA) in the sarcoplasmic reticulum (SR) membrane to regulate Ca\(^{2+}\) pump activity.\(^{46,47}\) After an electrical stimulus of the myocyte plasma membrane, Ca\(^{2+}\) is released from the SR allowing Ca\(^{2+}\) to bind to the myofilament protein troponin C, which then switches on the contractile machinery. For relaxation to occur, [Ca\(^{2+}\)] must decline, allowing Ca\(^{2+}\) to dissociate from troponin. Ca\(^{2+}\) is pumped back into the SR by the SERCA.\(^{48}\) Skeletal muscle MLN forms a single-pass transmembrane alpha helix that binds to SERCA in the SR membrane and regulates calcium handling. MLN ablation in mice significantly increased Ca\(^{2+}\) handling and improved exercise performance, showing that MLN is the predominant inhibitor of SERCA pump activity in adult skeletal muscle.\(^{45}\) Very recently, that same group discovered a putative muscle-specific lncRNA that encodes a peptide of 34 amino acids and that they named dwarf open reading frame (DWORF).\(^{49}\) DWORF is robustly expressed in heart tissue, but DWORF mRNA was downregulated in dilated hearts of calcineurin transgenic mice. DWORF was found to enhance SR Ca\(^{2+}\) uptake and myocyte contractility displacing the inhibitory peptides PLN, SLN, and MLN from SERCA. Peak systolic Ca\(^{2+}\) transient amplitude and SR Ca\(^{2+}\) load were significantly increased in myocytes from DWORF overexpression mice. The pacing-induced Ca\(^{2+}\) transient decay rate was significantly enhanced in myocytes aMHC-DWORF transgenic mice, indicating increased SERCA activity in muscle cells overexpressing DWORF. In contrast, slow skeletal muscle lacking DWORF showed delayed Ca\(^{2+}\) clearance and relaxation and decreased activity of SERCA.\(^{49}\)
The reason why these micropeptides were missed earlier is that it is very difficult to identify functional short ORFs in RNA transcripts. As bioinformatics tools improve, more micropeptides will most likely be found and characterized.

**Therapeutic potential of targeting lncRNAs**

Our understanding of lncRNA biology is still in an elementary stage and much more experimental work is needed. As described in Chapter 1, lncRNAs can exert different functions. They can regulate expression of genes located in close proximity (cis-acting) or target distant transcriptional activators or repressors (trans-acting).\(^{50}\) LncRNAs can act as molecular decoys and ‘sponge’ protein factors or competing endogenous RNAs (ceRNAs) for miR target sites.\(^{51}\) Third, lncRNAs can act as molecular guides by directing ribonucleoprotein complexes to specific chromatin targets.\(^{52}\)

In addition to miRs, lncRNAs also have demonstrated to play crucial roles during cardiac embryogenesis (summarized in Chapter 2) and heart failure (Chapter 6) and in the development of heart failure, as their expression levels were found to be dysregulated in cardiac disease (summarized in Chapter 2). Expectedly, researchers are developing ways to correct the abnormal expression levels of these particular lncRNAs. Functional natural antisense transcripts (NATs) typically originate opposite the sense strand of many protein-coding genes, often overlapping in part with mRNA, promoter and regulatory regions.\(^{53}\) Similar to miR antisense oligonucleotides, single-stranded oligonucleotides can be synthesized to strand-specifically block the interaction of the NAT with the corresponding sense strand gene mRNA, causing transcriptional de-repression of the gene. Brain-derived neurotrophic factor (BDNF) is normally repressed by a conserved noncoding antisense RNA transcript, BDNF-AS. Inhibition of this transcript was achieved by single-stranded, 16-nucleotide gapmers consisting of three LNA-modified DNA bases at each of the ends with ten unmodified DNA bases in the middle, allowing for RNaseH cleavage. This approach resulted in upregulated BDNF mRNA, altered chromatin marks at the BDNF locus, increased protein levels and induced neuronal outgrowth and differentiation both in vitro and in vivo.\(^{54}\) Similar to miR antisense oligonucleotides, it is important to introduce chemical modifications to promote metabolic stability and minimizing the length of the oligonucleotide to aid cellular uptake, while maintaining selectivity to minimize off-target activities.
Inhibition of lncRNA function by antisense oligonucleotides will face similar challenges as antisense miR oligonucleotides do (Chapter 3). Toxicity and off-target effects and delivery issues still have to be addressed. On the other hand, lncRNAs do open up some possibilities for strategies that are not applicable to protein targets. Targeting a specific lncRNA could upregulate an endogenous gene in a natural way. Inhibiting lncRNAs that regulate the expression of haploinsufficient genes could increase activity of the functional copy and result in normal functioning. Whereas miRs are translational repressors targeting mRNAs, lncRNAs primarily regulate chromatin in order to inhibit transcription. Furthermore, in contrast to miRs that can target hundreds of different mRNAs, cis-acting lncRNAs are gene-locus-specific. There are probably thousands of unexplored lncRNAs, whereas fewer miR species exist and those have been more comprehensively investigated. LncRNAs have been reported to show relatively low expression compared to mRNAs. If lncRNAs are indeed transcriptional repressors, this could indicate that a small number of lncRNA molecules is sufficient to demonstrate efficacy, creating a potential advantage of targeting lncRNAs at a lower dose than the standard oligonucleotides used for mRNA and miR knockdown.

Classifying lncRNAs into diverse functional classes will capacitate the design of therapeutics that possess a certain mode of action, such as the transcriptional derepression shown by antisense lncRNA oligonucleotides. Further functional genomic studies are required to establish the roles of lncRNAs. Large-scale inhibition and overexpression approaches in proper cell models, with subsequent detailed in vitro and in vivo characterization of selected functional candidates will shed more light on the complex world of lncRNAs.
References


