Review article

# Discovery and functional characterization of cardiovascular long noncoding RNAs

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## ABSTRACT

Recent advances in sequencing and genomic technologies have resulted in the discovery of thousands of previously unannotated long noncoding RNAs (lncRNAs). However, their function in the cardiovascular system remains elusive. Here we review and discuss considerations for cardiovascular lncRNA discovery, annotation and functional characterization. Although we primarily focus on the heart, the proposed pipeline should foster functional and mechanistic exploration of these transcripts in various cardiovascular pathologies. Moreover, these insights could ultimately lead to novel therapeutic approaches targeting lncRNAs for the amelioration of cardiovascular diseases including heart failure.

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## Contents

## 1. Introduction

Coronary artery disease is the most frequent cardiovascular disorder, and typically leads to acute coronary syndromes and myocardial infarction. Ultimately, heart failure (HF) develops, and is associated with a high probability of death. Unfortunately, increased risk factors, including detrimental life style choices, mean HF is evolving into a major global epidemic with residual morbidity and mortality projected

to increase over the coming decades [1]. Existing therapeutic approaches for patients suffering with HF include for instance inhibitors of the renin angiotensin system and beta adrenergic receptor antagonists, which target neurohormonal signaling pathways [2]. Although these therapies have improved the survival of HF patients, they fail to reverse or even halt the pathogenesis of heart failure. In light of this unmet clinical need, the elucidation of the precise molecular mechanisms controlling pathological remodeling and identification of novel therapeutic targets are required with the promise of identifying alternative approaches to prevent this prevalent and deadly disease.

In the adult heart, stress-dependent neurohormonal and hemodynamic signals induce a pathological remodeling process associated with cardiomyocyte (CM) hypertrophy and fibrosis, which leads to further myocyte death during the chronic phase of the response [3]. These combined processes culminate to induce contractile and structural failure, the hallmarks of HF. At the cellular and molecular levels, these neurohormonal and hemodynamic stressors activate a network of integrated signal transduction cascades that converge on a set of core cardiac transcription factors (TFs) [4]. These evolutionary conserved TFs (e.g. GATA4, NKX2.5, and MEF2) interact in a combinatorial manner at target cis-regulatory sequences (promoters and enhancers) to elicit specific gene expression programs [5]. However, the notion that the gene regulatory networks (GRNs) activated by cardiac stressors are primarily protein-based regulatory systems has proved to be premature. With the advent of ultra high throughput sequencing technologies, a number of important studies have demonstrated that these GRNs are under the control of a myriad of interleaved networks of regulatory non-coding RNAs (ncRNAs) [6]. These ncRNAs control numerous aspects of pathological GRN activity via a diverse array of regulatory mechanisms. Currently, the best characterized ncRNAs in the heart are the microRNAs (miRNAs). These small regulatory ncRNAs contribute to fine-tune the activity of the cardiac GRN post transcriptionally via targeting protein-coding transcripts for degradation, thereby implicating miRNAs as important stress-dependent modulators of the physio-pathological response in the damaged heart. In addition to miRNAs, recent screens have identified other regulatory classes of ncRNAs, namely long noncoding RNAs (lncRNAs) [7]. In the present review, we provide the reader with suggestion to identify and functionally characterize novel lncRNAs in the cardiovascular system.

## 2. Long noncoding RNAs

The broad term lncRNA is an operational term that refers to transcripts greater than 200 nucleotides in length, which, in addition, do not apparently encode a protein sequence [8]. This size threshold is a convenient but arbitrary one that excludes most known classes of small regulatory and infrastructural RNAs, including tRNAs, piRNAs and miRNAs. Importantly, although these transcripts are referred to as noncoding, a few lncRNAs have been found to encode micropeptides [9]. Most lncRNAs are expressed at relatively lower levels when compared to messenger RNAs (mRNAs), demonstrate a more restricted expression pattern and are typically found to be associated with ubiquitously expressed chromatin modifying complexes or splice factors [8]. In contrast, those that are expressed at levels comparable to mRNAs appear to be implicated as structural scaffolds for specialized nuclear domains. Some lncRNAs are also enriched in the cytoplasm, suggesting roles in regulating cellular functions in this compartment. Collectively, these diverse lncRNA characteristics support a complex landscape of RNA biology that remains largely unexplored in cardiovascular biology and in particular heart failure [10].

Initially, lncRNAs have been classified according to their location on the genome and orientation as compared to the closest coding gene. Intergenic lncRNAs are located in between two coding genes, not overlapping therefore with any coding sequences. They are usually referred to as long intergenic (or intervening) noncoding RNAs (lincRNAs). Enhancer-associated lncRNAs represent a subclass of intergenic lncRNAs. In contrast, intragenic lncRNAs overlap with coding genes. In this case, lncRNAs can be transcribed from the sense or antisense strand. Natural antisense transcripts (NATs) have been defined as overlapping antisense noncoding RNAs that regulate the expression of their opposite coding gene. Finally, bidirectional noncoding RNAs are transcribed at the close vicinity of a protein coding gene on the opposite strand. It is important to note that the subgroup of intergenic lncRNAs has been studied in priority since data are easier to interpret than those of overlapping lncRNAs. For a more detailed description of lncRNA biogenesis, evolutionary history, structure and function, we refer the readers to the recently published reviews [11–13].

Studies within the cardiovascular system are implicating lncRNAs as important transcriptional regulators of cardiac gene expression at the transcriptional and post transcriptional level. Table 1 lists a series of recently identified cardiovascular lncRNAs. Typically, lncRNAs exhibit distinctive roles in modulating tissue-specific epigenomic states and nuclear organization, which are critical for the transcriptional and epigenetic reprogramming that underpins HF pathogenesis [14]. The global reorganization of the nuclear architecture and epigenome is indeed central for the genome-wide transcriptional reprogramming that underpins pathological remodeling. Numerous characteristics render lncRNAs ideal molecules to provide cardiovascular cells with a catalog of molecular address codes to guide transcriptional and epigenetic regulatory events in the genome [15]. For instance, an important characteristic associated with lncRNAs in the cardiovascular system is that they exhibit richer tissue and cell specificity when compared to mRNAs and miRNAs [16,17]. These data suggest that an important function for lncRNAs is to dictate combinations of ubiquitously expressed chromatin modifying protein complexes in a highly cell specific manner. This allows the establishment of tissue-specific epigenomic states that determine cellular fate and behavior post stress. A canonical example is Myheart (Mhrt), a lncRNA encoded within the myosin heavy gene 7 (Myh7) locus [18]. This heart-enriched lncRNA is required for maintaining cardiac function in the stressed heart, and its stress-dependent repression leads to cardiomyopathy. Importantly, forced expression of Mhrt is sufficient to protect the heart from cardiac hypertrophy and subsequent contractile failure. At the molecular level, Mhrt controls the local epigenetic environment by acting as a molecular decoy and antagonizing the activity of Brg1, a chromatin remodeling factor known to be important for the transcriptional reprogramming that occurs in the failing heart. Interestingly, human MHRT is also depleted in patients suffering with cardiomyopathy, supporting a conserved role through evolution.

Furthermore, lncRNAs can efficiently and rapidly operate both in cis, at their site of transcription, and in trans at remote locations around the genome. For example, a class of lncRNAs derived from developmental and adult cardiac enhancers activates neighboring genes in cis via DNA looping and pause release mechanisms [14,19]. A significant group of lncRNAs, typically associated with canonical active promoter chromatin marks, act primarily in trans as recruiters or decoys for chromatin remodeling complexes and transcription factors to silence or activate specific gene expression programs [20]. These functions are as a consequence of a particularly high affinity of lncRNAs for RNA binding proteins such as components of the Trithorax (e.g. Fendrr) [21] and Polycomb complexes (e.g. Bvht) [22]. Other trans-acting cardiovascular lncRNAs have been shown to function as sponges for miRNAs, titrating the ability of these small ncRNAs away from their sites of action [23]. Furthermore, lncRNAs can also regulate mRNA splicing, translation or degradation through association with mRNAs or protein components of RNP complexes. Therefore, it is becoming increasingly evident that cardiovascular lncRNAs have functions in a diverse array of gene regulatory processes. However the field is still in its infancy and it is imperative that we develop a logical and flexible approach for discovery and annotation of high priority lncRNAs. This should foster their functional and mechanistic exploration in the context of cardiovascular disease. In this review, we summarize, discuss and propose best practices

**Table 1**
Cardiovascular lncRNAs.

| LncRNA | Species | Biotype | Function; association with disease | Mechanism | Ref. |
|---|---|---|---|---|---|
| *ALIEN* | Human | Intergenic | Cardiovascular commitment | Unknown | [25] |
| *ANRIL* | Human | Antisense | Metabolism; coronary artery disease | Unknown | [58] |
| *HIF1A-AS2* | Human | Antisense | Heart failure | Unknown | [59] |
| *KCNQ1OT1* | Human | Antisense | Cardiovascular development; arrhythmia | Guide | [60] |
| *LIPCAR* | Human | Unknown | Heart failure | Unknown | [39] |
| *MIAT* | Human | Intergenic | Myocardial infarction | Decoy | [61] |
| *MYL4-AS* | Human | Antisense | Cardiac hypertrophy | Unknown | [62] |
| *PUNISHER* | Human | Antisense | Endothelial cell identity | Unknown | [25] |
| *SENCR* | Human | Antisense | Smooth muscle contractility | Decoy | [26] |
| *TERMINATOR* | Human | Intergenic | Pluripotency; cardiovascular development | Unknown | [25] |
| *Bvht* | Mouse | Intergenic | Cardiac mesoderm commitment | Decoy | [22] |
| *Carl* | Mouse | Intergenic | Mitochondria; cardiomyocyte apoptosis | Decoy | [46] |
| *Chrf* | Mouse | Intergenic | Cardiac hypertrophy | Decoy | [23] |
| *Fendrr* | Mouse | Intergenic | Cardiac development | Guide | [21] |
| *Malat1* | Mouse | Intergenic | Endothelial cell identity | Decoy | [47] |
| *Mhrt* | Mouse | Intergenic | Cardiac hypertrophy | Decoy | [18] |
| *mm85* | Mouse | Intergenic | Enhancer-associated lncRNA | *Cis*-regulation | [19] |
| *Novlnc6* | Mouse | Intergenic | Enhancer-associated lncRNA | Unknown | [16] |
| *Smad7-lncRNA* | Mouse | Antisense | Enhancer-associated lncRNA | *Cis*-regulation | [19] |

involving state of the art technological and computational approaches for the discovery and characterization of cardiovascular lncRNAs. Exploring this uncharted territory has wide ranging implications for our understanding of cardiovascular biology and disease, representing a potential treasure trove of specific and efficient therapeutic targets for the amelioration of heart disease.

## 3. Discovery of cardiovascular lncRNAs

Gene expression profiling within the cardiovascular system typically utilizes the powerful approaches of massively parallel RNA sequencing (RNA-Seq). The pervasive nature of these approaches has recently led to an avalanche of data on cardiovascular transcriptional units, complexity and isoforms in a number of cardiovascular contexts [7]. RNA-Seq has now been used for a number of cardiovascular cell types, including cardiovascular precursor cells, cardiomyocytes, endothelial cells and vascular smooth muscle cells during differentiation and maturation, development and in adulthood including models such as myocardial infarction and pressure overload (Table 1) [16,19,24–28]. The first step in the analysis process is to assemble transcripts from the sequence reads. Classically, this step involves first mapping of the sequence reads to a reference genome and then assembling the transcripts based on the genomic coordinates using tools such as *Cufflinks* [29] or *Scripture* [30]. An alternative strategy is to perform transcript assembly directly from the sequence reads without the need of a reference genome (e.g. *Oases* [31], *Trinity* [32]). The latter strategy is necessary when no reference genome is available, or the reference genome is of poor quality. In our work, since we focus on identification of lncRNAs in mouse and human for which the genome sequences are well annotated, we have used a classical approach of first mapping RNA-Seq reads to the genome and then performing ab initio transcript reconstruction using the *Cufflinks* algorithm [16,19]. A workflow summarizing our lncRNA discovery pipeline is shown in Fig. 1. We start from three or more samples of polyA + mRNA and sequence to a depth of 200–400 million 100nt paired-end reads. At least three biological replicates per condition are necessary to enable any meaningful downstream statistical comparisons. It is favorable to sequence as many replicates as possible to gain enough power to detect differences genome-wide. The number of samples required will depend strongly on the biological and technical variability of an experiment. In our experience, we have found that, for exploratory analyses of homogeneous samples (e.g. cell lines, tissues from genetic mouse mutants and controls) in a highly controlled experiment, three biological replicates at high read depth can provide on the one hand sufficient reads to detect novel lncRNAs, and on the other sufficient power to detect statistical

differences between conditions. Of course, in a real world setting involving the use of clinical subjects, many more samples would be required to achieve a similar statistical power. Following transcript assembly from mapped sequence reads, the next step in our pipeline is to merge the results of the ab initio transcript prediction with known transcripts from a high quality database of known transcripts such as *Ensembl* [33]. This involves a comparison of the genomic coordinates of the RNA-Seq-generated transcripts with those of the reference transcripts, creating a single GTF (Gene Transfer Format) file containing all known and novel transcripts. This is performed using *Cuffmerge*, which is part of the *Cufflinks* package [29]. Using the information gained from known transcripts it is possible to now divide the transcripts into known and novel. Of course, at this stage, both protein-coding and noncoding transcripts are represented. It is therefore necessary to separate these transcripts using appropriate computational tools. Such tools can be divided into conservation-based (CSF and PhyloCSF [34]), which rely on evolutionary conservation of coding sequences to test whether a particular transcript is coding, and non-conservation based (CPC [35], CPAT [36], GeneID [37]), which rely on sequence feature-based classifiers. The former conservation-based methods are limited in that they will only reliably detect coding regions from known protein families, and not recently evolved protein coding genes. The latter non-conservation based methods are based on training classifiers to recognize particular features of coding sequences. These latter methods have the advantage that they do not require a sequence to be conserved across species to predict that it would be coding, only that the sequence shares some features with most coding sequences. Such machine learning approaches however often suffer from high type 1 (false positive) and type 2 (false negative) error rates, and can behave better or worse depending on whether the dataset being tested is similar to the data used to train the algorithm or not. In our pipeline, we filter the novel transcripts to remove those that contain a single exon or are below 200nt in length (these are the current accepted standards to define a lncRNA), and then use *GeneID* to score novel transcripts for their coding potential. Novel single-exon transcripts are removed because this subgroup is more likely to be contaminated with artifacts due to fragments produced during RNA-Seq. In order to define an appropriate score cutoff to optimally separate coding and noncoding transcripts, we recommend first running *GeneID* on all the known coding and noncoding transcripts for a given experiment, and plotting the score distributions of known coding versus noncoding transcripts. In our example from myocardial lncRNAs the coding score cutoff was taken at the intersection of the known coding and noncoding score distributions (Fig. 1).

In our experience, paired-end sequencing to a depth of at least 300 million reads is essential to detect novel tissue-specific lncRNAs [16].
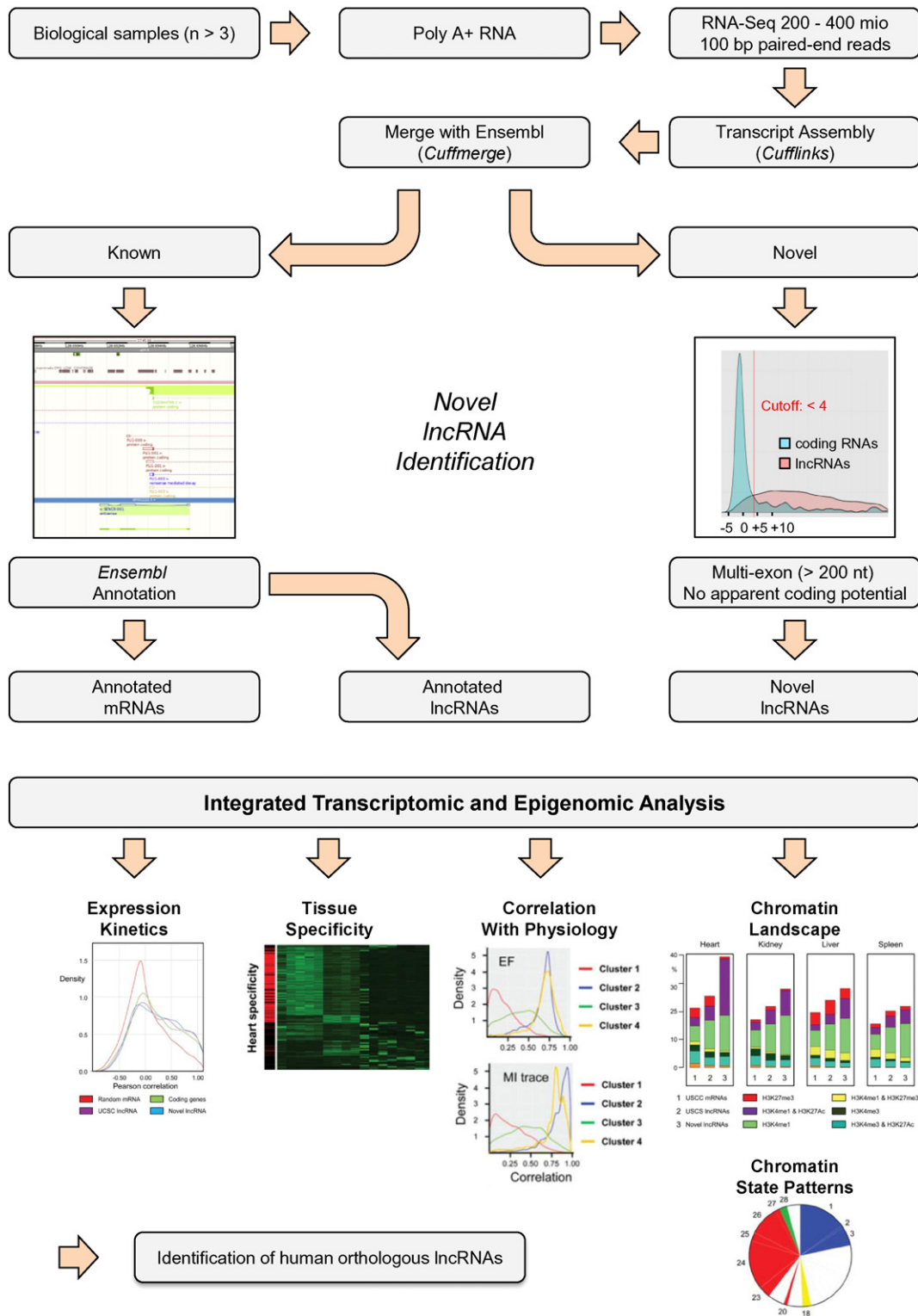
**Fig. 1.** Pipeline for the discovery and annotation of cardiovascular lncRNAs. See text for details. Images are reproduced with permission from Ounzain et al. EHJ. 2015 (Ref. [13]).

Using read depths of over 350 million paired-end reads, we were recently able to identify 1500 novel multi-exonic polyadenylated lncRNAs in the infarcted myocardium [16], and 2500 from mouse embryonic stem (ES) cell differentiating towards cardiomyocytes [19]. In order to test how important depth was in our discovery efforts, we performed a simulation in which we counted how many novel lncRNAs would have been discovered at increasing sequencing depths (Fig. 2; see legend for details). Our results are striking: If we had sequenced to a typical depth of 50 million paired-end reads, we would have missed 50–60% of the novel lncRNAs. It is important to emphasize again that the novel lncRNAs that required the greatest depth of sequencing for discovery were those exhibiting the most interesting functional and regulatory characteristics linked to cardiovascular cell function and biology [16]. In contrast, we have found that shallow sequencing will lead to the identification of mainly annotated, non tissue-specific lncRNAs. Based on this computational simulation, we suggest therefore that samples
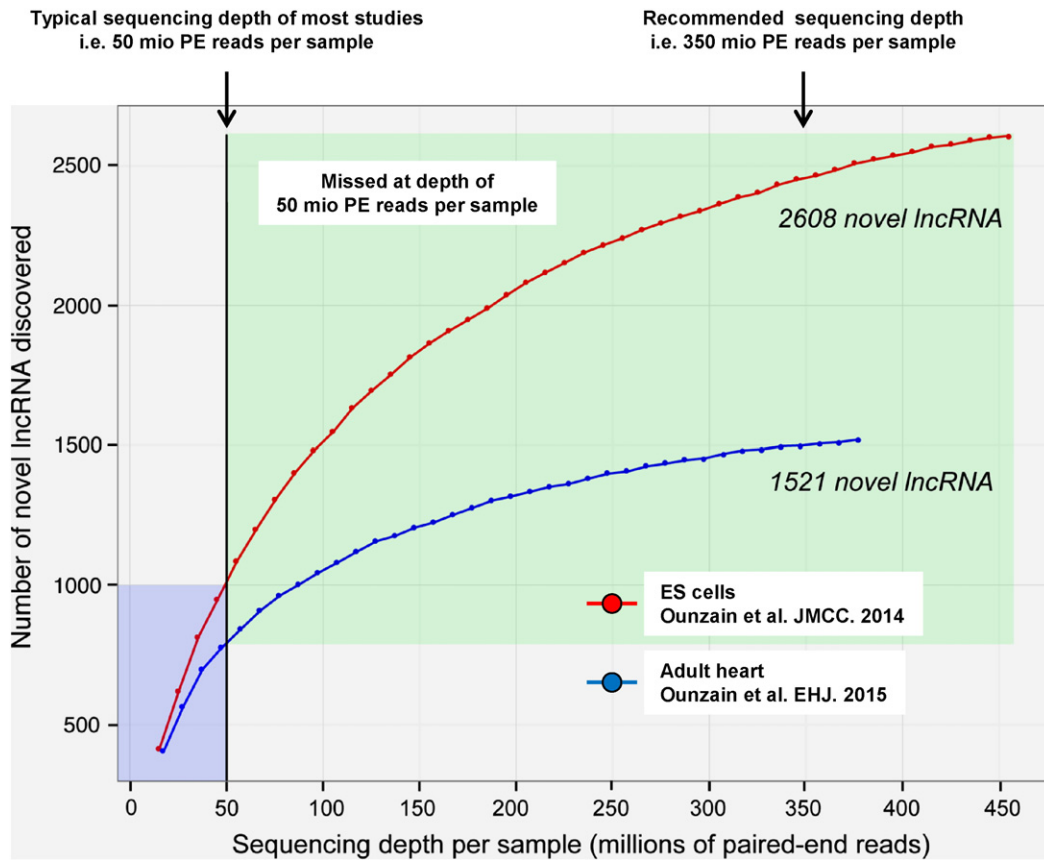
**Fig. 2.** Numbers of novel lncRNAs discovered at different read depths in adult heart and ES cells. Starting from the total reads in a dataset, 10 million reads were removed from the data iteratively, and the raw counts per gene were adjusted to the new total number of reads, by multiplying the counts by the proportion of total reads in the sample. An average per gene was then taken across all samples, and only those genes that passed our minimum threshold for expression were kept (mean counts ≥ 5) and plotted against read depth. For both the adult heart and ES cell experiments, the slope is steeper at lower read depths (up to 50 million reads), and tails off gradually at higher depths. Fifty million reads is the depth typically used in RNA-Seq experiments. The blue-boxed area highlights the numbers of lncRNAs that would be discovered using this standard read depth and below. The green-boxed area highlights the numbers of novel lncRNA that would be 'missed' when sequencing to this standard depth. Above 100 million reads, there is a steady increase in number of novel lncRNAs discovered as read depth increases, with no plateau reached even at read depths of 400 million and over. Based on this analysis, we predict that a plateau will be reached between 500 and 600 million reads per sample. We recommend sequencing to a depth of at least 350 million reads, which we predict will allow detection of the vast majority of novel lncRNAs in a sample.

should be sequenced at a depth of at least 350 million reads with a minimum of three biological replicates. In addition, the size of paired-end reads, i.e. 100 base pairs, is essential to facilitate transcriptome assembly and allow isoform resolution of both mRNAs and lncRNAs.

## 4. Functional annotation of cardiovascular lncRNAs

Once novel lncRNAs have been identified and computationally demonstrated to lack coding potential, candidates can be prioritized for functional characterization based on a number of criteria including, (a) tissue- and cell type-specific expression; (b) cytoplasmic vs. nuclear expression; (c) dynamic response to developmental or environmental cues; (d) guilt-by-association analysis, i.e. analysis of co-expression with coding genes of functional relevance; (e) correlated expression with cardiovascular physiological traits and parameters; (f) association with specific chromatin states; (g) inferred functions based on developmental chromatin state patterns and (h) the possible existence of a human ortholog [16]. To assess cell and tissue specificity, we recommend quantifying expression of newly identified lncRNAs in publicly available RNA-Seq datasets (e.g. ENCODE data). It is then possible to determine the relative expression of candidate lncRNAs in the tissue of interest, in this case heart, versus the mean expression in all non-cardiac tissues. This then allows heart specificity of new lncRNAs to be determined and interpreted. Not surprisingly, using comparable approaches, a number of studies have demonstrated that typically novel lncRNAs identified within the heart exhibit much greater cardiac specificity than protein coding genes and even miRNAs [16,17]. These findings do not rule out

that lncRNAs demonstrating more global expression pattern are not important for cardiac development and homeostasis. However, with regards to their therapeutic potential, heart-enriched lncRNAs represent ideal candidates since targeting these lncRNAs should minimize off-target effects.

As already mentioned above, many lncRNAs can remodel their local chromatin environment to regulate the expression of nearby developmental and physiological coding genes in cis to control homeostasis. Therefore, using a guilt-by-association approach to analyze the expression patterns of proximal coding genes represents a powerful way to identify functionally important cis-regulatory lncRNAs. Furthermore, lncRNAs whose expression kinetics are highly correlated with functionally important networks of coding genes during development or pathological remodeling also suggests such lncRNAs may be functionally involved in parallel processes. Depending on the cell type and tissue examined, this correlative approach can be expanded to identify lncRNAs highly correlated with physiological traits and characteristics. The unique nature of RNA-Seq approaches lend global lncRNA expression data amenable to direct correlation profiling with continuous physiological traits of choice. This is of particular relevance within the heart, an organ that can be physiologically assessed using non invasive methods including echocardiography to evaluate cardiac dimensions and function. For example, we and others have correlated lncRNA expression profiles with echocardiography derived physiological traits [16,17]. Impressively, novel lncRNAs were better correlated with cardiac traits when compared to mRNAs and miRNAs. Such an approach allows the filtering of lncRNAs predicted to be relevant in very specific

functional and remodeling-associated biological processes. From a biomarker perspective, this type of approach also demonstrates that unsupervised clustering of lncRNAs is able to distinguish physiological traits. It indicates that lncRNAs could represent highly specific indicators of physiological traits, with significant diagnostic and prognostic values in a clinical setting. Supporting this observation, recent investigations have supported the presence of lncRNAs in the blood. These molecules could aid in the diagnosis and prognosis of cardiovascular disease [38–40].

An increasingly powerful approach for the functional and regulatory annotation of novel lncRNAs is to characterize their underlying chromatin states in relevant tissue, cell types and pathological situations using publicly available and in house-generated ChIP-Seq data sets. Chromatin maps have previously been used for the initial identification of the intergenic class of long noncoding transcripts (lincRNAs), which were identified on the basis of their association with a canonical promoter signature (H3K4me3) at their transcriptional start sites. These intergenic lncRNAs typically encode trans-regulatory functions, regulating global transcriptional programs of numerous coding genes across chromosomes. Alternatively, a large fraction of newly identified lncRNAs, particularly in the heart, are derived from active tissue-specific enhancers characterized by H3K4me1 and H3K27Ac occupancy [16]. Enhancer-associated lncRNAs typically regulate their target coding genes in cis. Importantly, a number of studies have implicated these enhancer-associated lncRNAs as being functionally required for enhancer activity via mediating chromatin looping, promoter enhancer communication and Pol2 pause-release at target promoters [14]. We and others have identified hundreds of cardiac enhancer-associated lncRNAs that are involved in the global heart-specific enhancer-dependant cis-regulation of transcriptional programs, which underpin cardiac development and pathological remodeling. Supporting this, the proximal coding genes to these transcribed novel enhancer-associated lncRNAs are in general implicated in heart development and cardiac homeostasis. Moreover, enhancer-associated lncRNA expression in the injured heart usually correlates with coding gene expression [19].

Integration of chromatin state patterns and dynamics can also be leveraged for inferring functions for novel lncRNAs. This in particular pertains to chromatin state patterns associated with coding gene programs during cardiac differentiation. It has previously been shown that lineage-specific coding genes in differentiating cells cannot be assigned to a particular function based on their expression pattern alone. However, lineage-specific coding genes that are co-expressed at particular stages can be functionally grouped based on their distinct chromatin state patterns at their promoters during cardiac differentiation [24]. In other words, subgroups of co-expressed genes, clustered based on unique chromatin state patterns, appeared to be involved in highly specialized and distinct biological processes, including signaling,
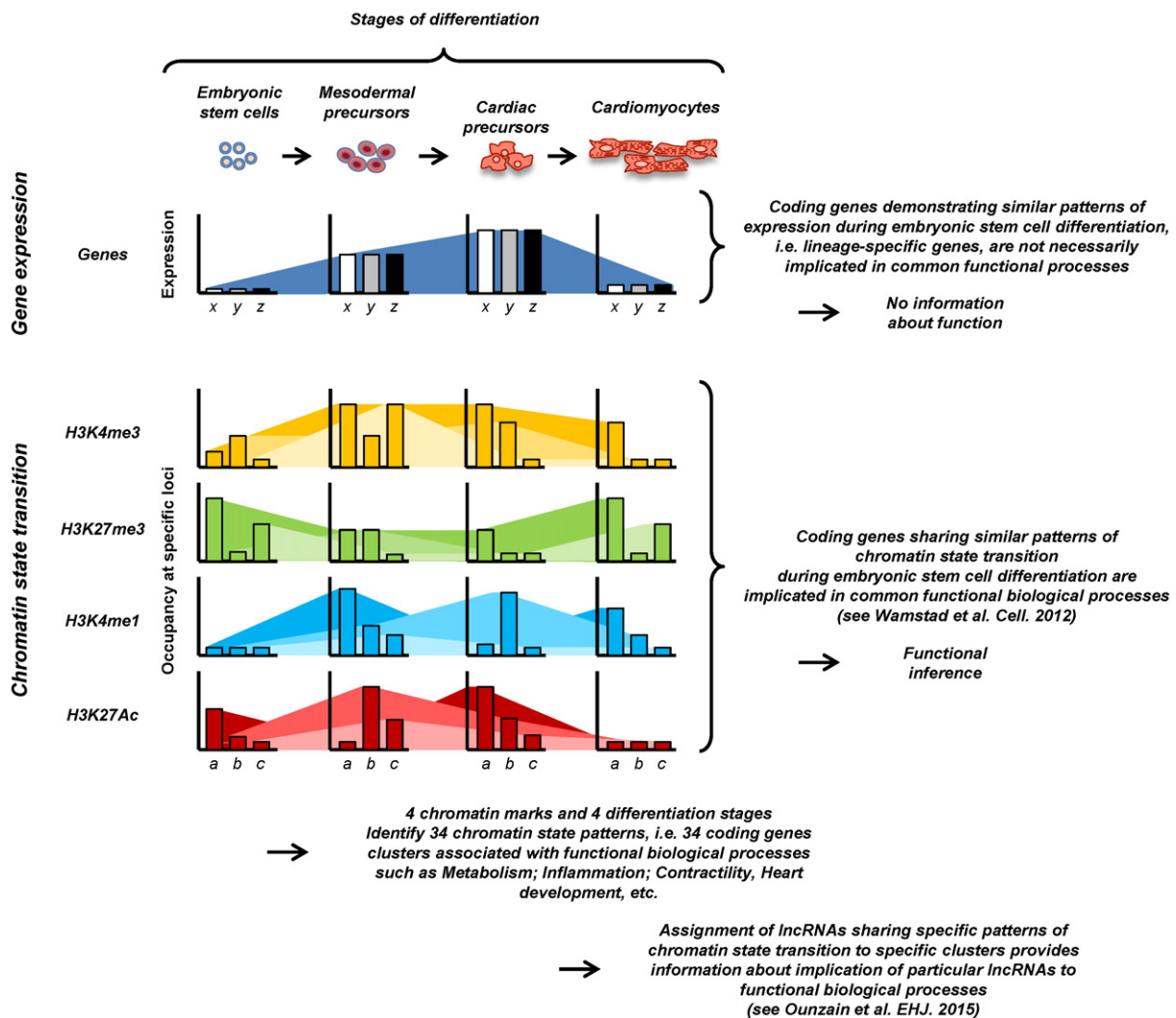


**Fig. 3.** Functional inference of novel cardiovascular lncRNAs based on chromatin state transition during cardiogenic differentiation. Using common patterns of chromatin state transition derived from ChIP-Seq data sets obtained in differentiating mouse ES cells (see Ref. [24]), it is possible to infer functions for novel cardiovascular lncRNAs.

metabolism and cardiac muscle contraction (Fig. 3). Using this information, one can assume that novel cardiovascular lncRNAs that shared comparable chromatin state patterns as those observed at coding genes, are likely to be involved in parallel biological processes. This approach provides a powerful unbiased chromatin-based proxy to infer functions and annotate novel cardiovascular lncRNAs. Using this approach, we found that heart-enriched lncRNAs identified in the infarcted myocardium were preferentially assigned to chromatin pattern clusters linked to specialized cardiac-specific processes, including heart development and contractility [16]. In addition, the novel lncRNAs, which, as described above, were identified using greater RNA sequencing depth, were more associated with these cardiac functional processes than annotated mRNAs and lncRNAs. These findings give further support to the relevance of the low-abundance lncRNAs for cardiac physiology. Inferring function during cardiogenic differentiation in ES cells makes possible the identification of novel lncRNAs that are expected to play significant roles in the adult heart. Moreover, this analysis can predict the functional processes that may be perturbed in vivo when experimental modulation of the lncRNA of interest is performed.

Upon completion of the integrated genomic approaches, it is important to identify human orthologs. This can be achieved directly by utilizing human heart RNA Seq data sets and mapping the lncRNA catalog to the human genome using TransMap, a cross species transcript alignment tool [41]. TransMap will map murine cardiovascular lncRNAs across the human genome using syntenic BLASTZ alignments that consider gene order and synteny. Furthermore, TransMap can integrate RNA sequencing reads from human samples to refine and complete ortholog identification and annotation. Finally, once orthologous human lncRNAs have been identified, it is possible to assess human lncRNA expression in human RNA Seq datasets, including those generated by large scale consortia such as the ENCODE [42] and Gtex consortiums [43]. It is also possible to map single nucleotide variants identified in genome wide association studies (GWAS) and linked to common cardiovascular traits and disease with orthologous lncRNA loci. This approach is likely to provide clues to the potential functions of identified cardiovascular lncRNAs. The first evidence for a putative role of lncRNAs in cardiovascular diseases came from a GWAS that identified a susceptibility locus for coronary artery disease on the human chromosome 9p21

[44]. This locus is in linkage disequilibrium with a lncRNA named antisense noncoding RNA in the INK4 locus (ANRIL) suggesting that single nucleotide variants within ANRIL could contribute to susceptibility for the development of coronary artery disease. Indeed, a number of follow-up studies have demonstrated that ANRIL expression is indicative of a risk for peripheral artery disease, carotid atherosclerosis, coronary atherosclerosis and other vascular diseases [45].

## 5. Functional assessment of cardiovascular lncRNAs

Prior to functional dissection, it is imperative that candidate lncRNA expression profiles are validated by quantitative methods including RT-PCR in relevant tissue samples and possibly in isolated primary cells, to gauge expression levels and tissue and cellular specificity. Moreover, in situ hybridization can be executed on appropriate histological tissue sections to confirm expression data. To better understand the physiological roles of cardiovascular lncRNAs during development and disease, and to decipher whether their regulatory functions operate via cis or trans mechanisms generally requires experimental modulation of lncRNA expression. These include in vitro and in vivo gain- and loss-of-function approaches (Fig. 4). Initially, cardiovascular lncRNA knockdown has been performed using RNA interference, such as small interfering (siRNA) or small hairpin RNAs (shRNA), followed by molecular and cellular analysis to determine the global effects on gene expression and cellular behavior. In a seminal study, this approach was used to identify and characterize a lncRNA important for cardiac specification, which was named Braveheart (Bvht) [22]. Transducing mouse ES cells with shRNAs to deplete Bvht led to impaired cardiogenic differentiation in vitro. Through transcriptomic analysis, it was shown that Bvht acts upstream of the key cardiogenic TF, Mesp1, to induce its expression and direct correct temporal and spatial activation of the subsequent cardiac gene regulatory network. A similar approach was used for the characterization of previously unannotated human vascular lncRNAs, TERMINATOR, ALIEN and PUNISHER [25]. These lncRNAs were specifically expressed in pluripotent stem cells, cardiovascular progenitors and differentiated endothelial cells. Loss of function using shRNAs in vitro and antisense morpholino nucleotides against zebrafish orthologs in vivo demonstrated that all three lncRNAs are involved in vertebrate



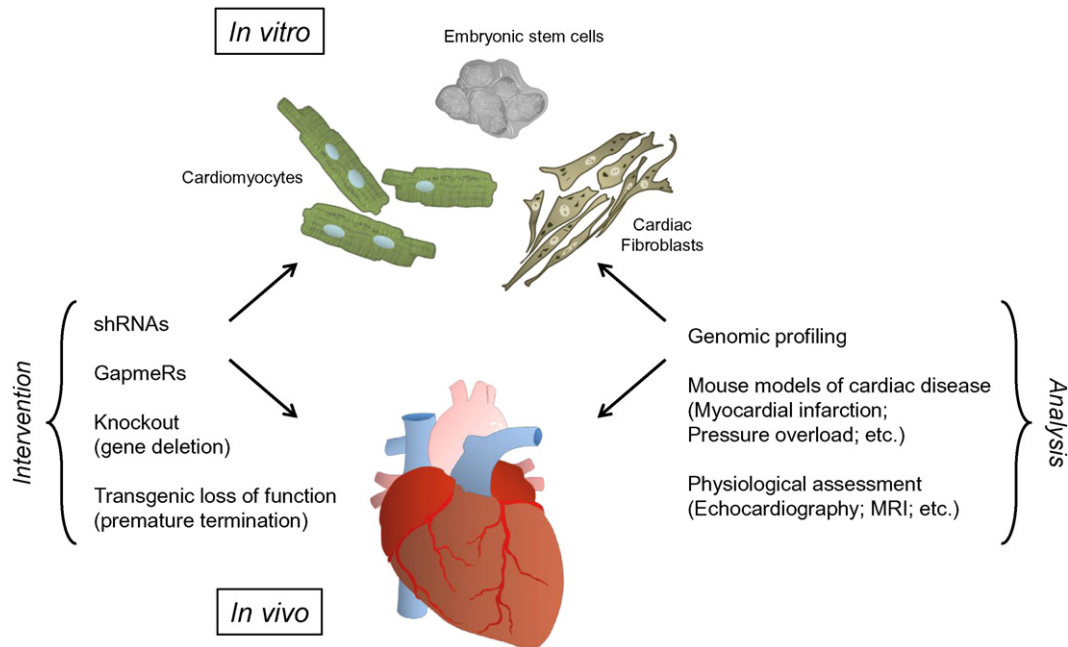Fig. 4. Different approaches for the functional assessment of lncRNAs in vitro and in vivo. Gain- and loss-of-function approaches using shRNAs, modified antisense oligonucleotides and classical genetic models can be adopted in vivo and in vitro to assess novel cardiovascular lncRNA function. Once manipulated, downstream genomic and functional consequences can be analyzed using various techniques and physiological models.

cardiovascular development. In addition to endothelial cells, siRNA approaches have also been successfully adopted in smooth muscle cells (SMCs). For example, siRNA mediated knockdown of the SMC lncRNA *SENCR* results in deregulation of a SMC-specific gene, namely *MYOCD*, which is a critical co-factor responsible for dictating the SMC gene program [26]. Finally, shRNA was also used to deplete the cardiomyocyte-enriched lncRNA, *Carl*. This lncRNA acts as a competitive endogenous RNA (ceRNA) for miR-534, which regulates mitochondrial fission and cardiomyocyte apoptosis [46].

Despite the success of RNA interference strategies, concerns have been raised about the efficacy of these techniques for depleting nuclear, and in particular, enhancer-associated lncRNAs. Indeed, the machinery used by the RNA interference pathway is predominantly enriched within the cytoplasm. To overcome these obstacles, and allow targeting of nuclear-enriched cardiovascular lncRNAs, many investigators are utilizing modified antisense oligonucleotides (ASOs), called LNA GapmeRs. Importantly, GapmeRs degrade transcripts in the nuclear compartment via a RNaseH-dependent mechanism, leading to efficient depletion of nascent transcripts. This has proven to be particularly efficient for the depletion of cardiac enhancer-associated lncRNAs in cardiomyocytes (e.g. *Novlnc6*), cardiac fibroblasts (e.g. *Smad7-lncRNA*) and cardiac precursor cells (e.g. *mm85*) [16,19]. Therefore, LNA GapmeR-mediated depletion of these enhancer-associated cardiovascular lncRNAs results in perturbed expression of the target coding genes. GapmeRs also offer a powerful approach for depleting lncRNAs in vivo. *Malat1* is a lncRNA predominantly enriched in the nucleus and highly responsive to hypoxia in human umbilical vein endothelial cells. Inhibition of *Malat1* in vivo using LNA GapmeRs impaired vascularization of the retina and ischemic hind limb [47]. This identifies this transcript as an interesting cardiovascular target to promote or block angiogenesis. More generally, involvement of candidate lncRNAs in specific biological processes can be evaluated in large-scale assays based on the unbiased screening of large collections of GapmeRs in relevant cell types using high-resolution techniques.

In addition to using RNA interference and modified antisense oligonucleotide approaches, genome editing technologies can be used to modify cardiovascular lncRNA expression in vivo. Classical approach via generating transgenic knockouts, either germline or conditional knockouts, can be envisaged. However, we are facing two main constrains. First of all, deletion of lncRNA gene overlapping a coding gene might be largely uninformative due to possible interference with natural expression of the coding RNA. Second, deletion of any given DNA sequence to eliminate the corresponding lncRNA might produce a phenotype solely because a mutation is introduced in a functional DNA element. In a prominent study, the depletion of *Fendrr* was therefore obtained by inserting a premature transcription stop signal into the first exon [21]. This resulted in embryonic lethality as a consequence of impaired cardiac development and body wall formation. Mechanistically, *Fendrr* orchestrates epigenomic states critical for cardiovascular cell specification and differentiation. Using a different genetic approach, the *Fendrr* locus was replaced by the LacZ reporter gene, resulting also in embryonic lethality, however much later during development [48]. Follow-up studies suggested that *Fendrr* loss of function led to defects in lung maturation and vascularization. In the future, the lncRNA field will certainly benefit from new techniques to generate targeted mutations in the genome, such as the TALEN and Cas9/CRISPR systems. Transgenic approaches for gain-of-function studies have also gained traction and illuminated roles for newly described lncRNAs. As described earlier, *Mhrt* is an antisense transcript to the Myh7 locus and was recently shown to interfere with cardiac hypertrophy and subsequent development of heart failure. This was formally demonstrated using a transgenic approach for restoring normal *Mhrt* levels in the stressed heart, which blunted the switch from Myh6 to 7 expression and protected the heart from pathological remodeling [18]. This effect was mediated by the binding of *Mhrt* to the Brg1/Baf chromatin remodeling complex, titrating it away from its target genomic loci. Although

gain-of-function approaches reveal important trans-dependent regulatory roles for cardiovascular lncRNAs, loss-of-function approaches currently represent the most biologically relevant strategy to investigate underlying molecular mechanisms. First of all, many lncRNAs act in a dose-dependent manner when binding ubiquitously expressed chromatin modifying protein complexes. As such, overexpression of candidate cardiovascular lncRNAs could lead to spurious effects through dominant-negative actions. Specifically, supra physiological lncRNA expression could titrate important chromatin modifying complexes away from their natural genomic sites of action, leading to unpredictable effects. Finally, a large fraction of newly discovered cardiovascular lncRNAs act as regulators of the epigenome and nuclear architecture in cis at their site of production. In particular, the regulatory function of lncRNAs derived from active enhancers act primarily at their endogenous site of production. Therefore, modulation of the nascent transcript is critical and exogenous overexpression is not a viable option.

## 6. Mechanistic assessment of cardiovascular lncRNAs

Complete characterization of cardiovascular lncRNAs requires a detailed knowledge of the molecular mechanisms associated to their function. Two recently developed techniques provide a means to assess when and where lncRNAs are binding and acting within the genome. These comparable techniques, namely chromatin isolation by RNA purification (ChIRP) [49] and capture hybridization analysis of RNA targets (CHART) [50], are able to map the genomic binding sites of endogenous lncRNAs. CHART uses cocktails of several short, complementary and affinity tagged oligonucleotides. These complimentary oligonucleotides allow specific enrichment for endogenous lncRNAs, along with their genomic DNA targets from reversibly crosslinked chromatin extracts, which can be sequenced to identify genome-wide binding sites. Similarly, lncRNA-binding proteins can be identified by CHART mass spectrometry. ChIRP also hybridizes biotinylated complimentary oligonucleotides to crosslink chromatin, although in this case tiling oligonucleotides are used, which require no previous knowledge of lncRNA structural domains. ChIRP can also be combined with DNA sequencing and mass spectrometric approaches to identify genome wide binding sites and protein partners.

Not all cardiovascular lncRNAs interact with chromatin but the vast majority of functional lncRNAs bind to and recruit protein complexes. The identification of the lncRNA interactome is thus critical for a complete understanding of cardiovascular lncRNA function. A conventional approach to address this question relies on the use of RNA chromatography [51]. Here, one can use affinity tagged oligonucleotides spanning the lncRNA of interest as baits on which interacting proteins can assemble. Interacting proteins are then eluted and analyzed by mass spectrometry (MS). Once potential interacting protein partners have been identified, classical approaches can be used including RNA-immunoprecipitation assays followed by qRT-PCR to validate such interaction. However, this classical approach can often generate false positives due to promiscuous binding of proteins to the oligonucleotide baits. Direct capture of endogenous lncRNAs using antisense oligonucleotide probes as with ChIRP and CHART MS can reinforce the relevance of the identified proteins. For instance, purification of endogenous interacting partners has been demonstrated for the human telomerase RNA TERC and the miRNA let-7 [52,53]. However, a major challenge is that proteins, unlike DNA or RNA, cannot be amplified for further validation. This places an experimental constraint on the amount of material required for in vivo purification due to the lower copy number of lncRNAs. Newly developed technologies, including protein arrays, can be used to screen RNA binding proteins without the obvious constraint in starting material. Microscale devices containing proteins printed on a solid support, allow the binding of synthesized RNAs containing a fluorescent label [54,55]. Following specific binding to proteins, the array will illuminate their corresponding coordinates. This can be imaged in

a similar way to nucleic acid microarrays and interacting proteins can be identified in a high throughput manner.

In addition to proteins, lncRNAs can also interact with RNA cofactors. RNA interactions can be assayed by hybridization techniques, including ChIRP. Using this approach bound RNAs are eluted and subjected to RNA sequencing or quantitative RT-PCR. Investigators have demonstrated that a lncRNA named TINCR, known to control epidermis differentiation, interacts with mRNAs implicated in skin development, impacting their expression [56]. Alternative methods use alternative crosslinking approaches, in particular UV light, which are able to capture only hybridizing RNA molecules. For example, crosslinking, ligation and sequencing of hybrids (CLASH) has been successfully used to examine direct RNA pairing between miRNAs and their target mRNAs, and can be envisaged for lncRNAs in the not too distant future [57].

## 7. Conclusion

We are entering a new era where we are beginning to understand the complex roles of cardiovascular lncRNAs during development and disease. This highly integrated and complex layer of regulatory noncoding RNAs exhibits dynamic interplay with components of various protein complexes to dictate the activity of the cardiac gene regulatory network. Cardiac pathological states, including heart failure, are intimately linked to the dynamics of cardiovascular lncRNA expression and regulatory functions. To increase our understanding and potentially identify new avenues for therapeutic intervention, the full extent of the cardiovascular lncRNA transcriptome needs to be determined and correctly annotated. This needs to be executed at different stages of cardiac development and during disease. Genomic approaches should in the future also interrogate the non-polyadenylated and single exon RNA fraction (i.e. eRNAs). This should open up new insights into the biology of cardiovascular cells, and hopefully will identify new players in the regulatory network governing cardiac development and homeostasis. Of critical importance is that investigators discovering new cardiovascular lncRNAs leverage the plethora of new approaches to decipher the language used by lncRNAs to interact with their respective protein or RNA partners. This should illuminate our understanding of these still elusive but potentially exciting therapeutic targets.

### Author contribution

SO and MI wrote the paper; FB and TP prepared the figures; and TP edited the manuscript.

### Disclosure statement

A patent application covering the therapeutic use of lncRNAs has been filed, and is pending. PCT application PCT/EP2014/078868 filed on December 19, 2014, based on US provisional Patent application No. US 61/964,591 filed on December 20, 2013, naming as inventors S. Ounzain and T. Pedrazzini.

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