REVIEW

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Interplay of chromatin modifications and non-coding RNAs in the heart

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Abbreviations: EZH2, enhancer of zeste homolog 2; ncRNA, non-coding RNA; lncRNA, long non-coding RNA; MHC, myosin heavy chain; HDAC, histone deacetylase; HAT, histone acetyltransferase; miRNA, MicroRNA; NAT, natural antisense transcript; PcG, polycomb-group; AS, antisense; SET, Su(var)3-9 and enhancer of zeste; bdP, bi-directional promoter

Precisely regulated patterns of gene expression are dependent on the binding of transcription factors and chromatin-associated determinants referred to as co-activators and co-repressors. These regulatory components function with the core transcriptional machinery to serve in critical activities to alter chromatin modification and regulate gene expression. While we are beginning to understand that cell-type specific patterns of gene expression are necessary to achieve selective cardiovascular developmental programs, we still do not know the molecular machineries that localize these determinants in the heart. With clear implications for the epigenetic control of gene expression signatures, the ENCODE (Encyclopedia of DNA Elements) Project Consortium determined that about 90% of the human genome is transcribed while only 1-2% of transcripts encode proteins. Emerging evidence suggests that non-coding RNA (ncRNA) serves as a signal for decoding chromatin modifications and provides a potential molecular basis for cell type-specific and promoter-specific patterns of gene expression. The discovery of the histone methyltransferase enzyme EZH2 in the regulation of gene expression patterns implicated in cardiac hypertrophy suggests a novel role for chromatin-associated ncRNAs and is the focus of this article.

Introduction

The mammalian heart is the first organ to form in the vertebrate embryo. During development, heart chambers undergo structural changes mediated by specific cellular and extracellular cues such as hormone stimulation. Heart development involves stage-specific changes that are precisely regulated by spatial and temporal events on chromatin to regulate specific gene expression patterns.[1] For example, genes expressed at later stages in cardiac development, such as cardiomyocyte maturation and terminal differentiation, show mono-methylation of histone H3 lysine 4 (H3K4me1) at early stages of development, whereas activation at later stages are often specified by H3K4me3 modification.[1] During lineage commitment there are stage-specific acetylation (H3K27ac) and methylation (H3K4me1, H3K4me3 and H3K27me3) of lysine residues on histone H3 regulating gene expression and cardiac differentiation. For example, both methylation and acetylation of histone proteins at distinct lysine positions determine specific histone modification signatures that predict gene expression patterns that serve as transcription-factor binding sites as well as the exchange of co-regulatory complexes on promoters.[2] Gene activation in pluripotent stem cells is associated with H3K4me1 patterns at gene promoters, which are also activated at later stages in the cardiac lineage, which is in striking contrast to H3K27me3 patterns and genes destined for suppression. Genes that code for the adult isoform cardiac contractile protein such as α-myosin heavy chain *(α-MHC)* and the transcription factor *NKX2.5*, are activated specifically at later stages of cardiac differentiation. These genes show high levels of H3K27me3 deposition at pluripotent stage, which are gradually erased and replaced by H3K4me3 modification.[3]

Cardiomyocyte cells respond using adaptive mechanisms to changing environmental stimuli such as increased workload. Such physiological changes are marked by an increase in cardiomyocyte size and ventricular mass, which is referred to as cardiac hypertrophy. Chronic exercise training or pregnancy can increase heart muscle mass and contractile ability, often referred to as physiological hypertrophy.[4] However, there is a fine balance between physiological and pathological hypertrophy which are distinguished by cardiac failure. Pathophysiological surroundings such as acute and chronic myocardial stress including hypertension, valvular disease, and myocardial infarction, can dramatically increase the size of the ventricular chamber.[4,5] This is referred to as pathological-cardiac hypertrophy and, like physiological hypertrophy, stimulates a phase of neurohumoral and biomechanical signals within the myocardium. While it is considered that physiological hypertrophy is generally advantageous as well as reversible, pathological hypertrophy causes irreversible remodeling leading to deformation of the ventricles and reduced heart contractility.[6]

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The discovery of specific activator and repressor complexes important in cardiac development has revealed several mechanistic insights into myocardial function, cardiac development as well as heart disease. Ventricular hypertrophy is associated with re-activation of fetal genes that include *ANP*, *BNP*, and β-*MHC* as well as the suppression of *SERCA2a* and α-*MHC* genes in the adult heart.[6] The recruitment of ATPase-dependent chromatin remodeling complexes that belong to the SWI/SNF family[7] have been shown to contextually associate with either histone acetyltransferases (HATs) or histone deacetylases (HDACs) to regulate cardiac gene expression.[8] Indeed, the recruitment and binding of p300 HAT enzyme on gene promoters is closely associated with chamber-specific gene expression patterns conferred by histone acetylation under physiological states.[9] In addition, recent studies have expanded the complexity of regulatory determinants that participate in cardiac gene function, for example histone modifying proteins such as EZH2 and ASXL2 specify *MHC* gene expression in postnatal cardiac homeostasis.[10,11] Human homologs of *Drosophila* genes (Enhancer of zeste homolog 2 and Additional sex combs-like protein 2) EZH2 and ASXL2 are members of the Polycomb group (PcG) protein family implicated in maintaining gene repressive states by chromatin modification during later stages of heart development.

Mechanisms that regulate gene expression are under the direct control of specific classes of transcription factors and core machinery that serve to alter chromatin structure and function. However the precise actions of transcription factors and chromatin remodeling determinants, including histone and non-histone modifying enzymes in gene transcription are poorly characterized in the heart. Moreover, the diversity of transcription factors and chromatin modifying enzymes specifying gene expression patterns presents a major conceptual problem when attempting to predict specific interactions with target genes. In this article we explore the basis of cell type-specific and gene-specific patterns of gene regulation that integrate chromatin-interacting ncRNAs with histone modifying enzymes that functionally serve to alter gene structure and expression. Recent experimental observations show that chromatin remodeling and histone modification confer important transcriptional programs as a result of development and cardiac disease.[12-15] The diverse interplay of histone modifying enzymes interacting with long non-coding RNAs (lncRNA) that serve to localize DNA-binding proteins as well as direct specific post-translational modifications to regulate gene expression has been described and is the focus of our discussion.[16-18]

Physiological Roles of lncRNAs in the Heart

Recent advances in nucleic acid sequencing technologies have revealed that nearly 90% of the genome is transcribed in one tissue type or another, with estimates that between 70–98% constitute ncRNAs.[19-21] These transcripts are broadly classified in two groups according to nucleotide length: short ncRNAs (<200 nt), such as microRNA (miRNA) and long ncRNAs (>200 nt), such as the natural antisense transcripts (NATs) (**Table 1**). Interestingly, ncRNAs have been thought for some time to interact with DNA to regulate important nuclear functions. Indeed, Jacob and Monod explored this concept of base complementarity between RNA and DNA sequences[22] which later was experimentally examined in triplex-forming sequences derived from human c-MYC.[23] Direct evidence of interacting ncRNA mediating gene silencing-epigenetic changes exposed recruitment of important regulatory components in RNA-dependent DNA methylation.[24]

Table 1. Classification of functional ncRNAs. Transcriptional gene silencing functions of short (grey background) and long ncRNAs by chromatin interaction

ncRNA class	Chromatin interaction
MicroRNA (miRNA)	Yes[114]
Small interfering RNA (siRNA)	Yes[115]
Piwi-interacting RNA (piRNA)	Yes[116]
Small nuclearRNA (snRNA)	Yes[92]
Small nucleolarRNA (snoRNA)	Yes[117]
Natural antisense transcript (NAT)	Yes[80]
Large intergenic ncRNA (lincRNA)	Yes[35]
Promoter associated RNA (paRNA)	Yes[118]
Circular RNA (circRNA)	Yes[78]
Enhancer RNA (eRNA)	Yes[119]
Pseudogene RNA (trans-NAT)	Yes[120,121]
Transcribed ultraconserved regions (T-UCRs)	Yes[122]
Short-lived RNA transcripts (SLiTs)	Yes[32]
Telomeric repeat-containing RNA (TERRA)	Yes[123]
Transfer RNA (tRNA)	Not reported
Ribosomal RNA (rRNA)	Not reported

When, in 1993 two studies published back-to-back in *Cell* described a putative role for short ncRNAs in *C. elegans* development, the importance of these critical findings was probably underappreciated in transcription biology.[25,26] How ncRNAs recognize and interact with target sequences to regulate gene expression still remains poorly characterized. Although short ncRNAs are strongly conserved but of unknown function, the seminal discoveries by the groups led by Ambros and Ruvkun have revealed a regulatory complexity mediated by ncRNAs. The field has expanded tremendously with a better understanding of the significance in biology and disease. Recent studies now show that during development, ncRNAs are expressed in a dynamic fashion and regulated by specific cellular and environmental cues.[16,17]

The importance of short ncRNAs in heart development was elegantly demonstrated by cardiac-specific deletion of miRNA-processing enzyme, DICER.[27] Abundantly expressed in the heart, *miR-1* and *miR-133* are associated with cardiovascular development and myeloid differentiation.[28-30] Recently, functional paradigms for several lncRNAs have also been described such as the participation in embryonic differentiation and cell-lineage development as well as transcriptional control.[16,17,31,32] While

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Table 2. Chromatin immunoprecipitation in mouse left ventricle shows specific interaction of EZH2 at genes with bi-directional transcription.

Gene
Ink4a, Ink4b, Ak148321/ANRIL
Pax6, Pax6ost1
Nppa, Nppa-as1
Miat, 1700028D13Rik
α-MHC, β-MHC, AS β-MHC
Foxd2, 9130206I24Rik
Hoxc11, Hoxc12, Hotair
Gata3, 4930412O13Rik
Dio3, Dio3os
Ucn, Ucn-as
Islr2, 1600029o15Rik
Dll4, Gm14207
Pou3f3, 2610017I09Rik
2610100L16Rik, Gm10724
Hoxa4, Hoxa5, Hoxa6, Hoxa7, 2700086A05Rik
Irx5, 4933436c20Rik
Fbxo44, Fbxo2
Otx2, Otx2os1
H2-K2, AA388235
Pcnxl2, Bc021891
Dlx6, Dlx6as-1
Tbx2, 2610027K06Rik
Myl4 (ALC-1), Myl4-AS
cTn1 (Tnnt3), cTn1-AS
Tgfβ3, Tgfβ3-AS

Listed are genes as enriched by ChIP using antibodies that recognize EZH2 and H3K27me3 modification.[52] Genes on sense and antisense strands are distinguished by an underline. A significant proportion of the genes enriched by EZH2-ChIP in the mouse heart show specific binding of EZH2 at key cardiac genes with antisense RNA expression. Several cardiac genes with antisense RNA expression including the cardiac regulatory lncRNA genes *ANRIL*, *MIAT,* and *NPPA-AS* appear to be bound by EZH2. Genes encoding non-cardiomyocyte expression programs such as the *PAX6*, which expresses opposite strand transcript is also repressed by direct binding of EZH2 in the heart. Increased expression of Myosin light chain (*MYL4*) and *TGFβ-3* genes was observed in EZH2 deficient mice,[10,52] both of which are known to express regulatory antisense transcripts, however, show no direct association of EZH2 at these promoters.[52]

lncRNAs can serve as spliceosome and ribosome components in eukaryotic RNA metabolism, recent experimental observations indicate a role in organizing chromatin conformation and shaping the genome. For example, chromatin interacting lncRNAs were recently identified as key determinants of gene imprinting (such as *XIST* and *KCNQ1OT1* as well as *AIR*), whereas the recruitment of PRC2 components are implicated in gene suppression events that involve *HOTAIR* and *TUG1*.[33-35] Recently, knockdown of lncRNAs expressed in embryonic stem cells has revealed more than one hundred functional lncRNAs associated with the maintenance of pluripotency.[36] In addition, several lncRNAs have been implicated in normal heart physiology. For example, in the mouse, *Braveheart* (*Bvht*) and *Fendrr* are thought to have critical roles in cardiac lineage specification during embryonic development.[16,17] The silencing of *Bvht* in mES cells results in the loss of cardiomyocyte beating in embryoid bodies (EB) at day 11 of differentiation.[16] Whereas the expression of tissue-specific *Fendrr* is a regulator of heart and body wall development.[17] While these results are not fully understood, it is hypothesized that *Bvht* and *Fendrr* control gene expression by interacting with the regulatory cofactors, PRC2 and TrxG/MLL complexes. These studies highlight the importance of lncRNA transcripts defining chromatin structure and gene expression necessary for heart development. Recent studies have also identified putative roles for over expressed lncRNAs in cancer (*MALAT1* and *HOTAIR*) and Alzheimer disease (*BACE1-AS*), as well as reduced expression of lncRNAs in anemia (*LincRNA-EPS*) and Huntington disease (*HTT-AS*).[37-40] In addition to the general involvement of DNA-binding motifs that function in the recruitment of transcription factors, new roles for lncRNAs in mediating chromatin-protein interactions have recently been described.[20,41] Several lncRNAs have putative sequence motifs and structural domains implicated in protein association and interacting with specific gene targets. Indeed, several chromatin-interacting proteins have recently been described to have ncRNA-binding domains such as the polycomb-group (PcG) proteins, which are involved in the suppression of gene expression mediated by chromatin modification.[42,43]

Non-Coding RNAs Connect EZH2 with Chromatin

The expression of lncRNAs and natural antisense transcripts have recently been shown to regulate gene transcription and protein translation in the heart.[14,15] The antisense (AS) transcripts to *NPPA* (*AS-NPPA*) and *β-MHC* (*AS-β-MHC*) are examples of regulatory lncRNAs in the myocardium. These transcripts are thought to associate with chromatin and regulate the expression of sense counterparts, *NPPA* and *β-MHC* whose expressions are regulated by EZH2 in the heart. The EZH2 lysine methyltransferase has a binding domain that is thought to mediate interaction with lncRNAs.[42] For instance, phosphorylation of threonine (T365) of EZH2 interacts with *HOTAIR* and *XIST*.[33] Although well characterized in cancer, the specific interactions of ncRNAs with histone modifying determinants such as EZH2 remain poorly described in the heart.[44] Several lysine methyltransferase proteins have a conserved SET-domain region, which is thought to be critical to chromatin association as well as enzymatic activity. A number of methyl-writing SET-domain family members such as G9A, SET7, SMYD3, SET2, SET1, and EZH2, can bind to single-stranded DNA and RNA.[45-48] In addition, several MLL family proteins that contain the SET-domain are known to interact with ncRNA either directly or indirectly.[49,50] The methyl-erasing enzyme, LSD1, is thought to bind directly to the 3′ end of the *HOTAIR* lncRNA to regulate *HOXD* gene expression.[51]

Recent data published by several groups suggest putative roles for antisense transcripts in mediating EZH2 interactions

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with chromatin (**Table 2**).[10,52] The expression of genes encoding contractile proteins and transcription factors implicated in heart disease are altered in EZH2-knockout mouse models.[10] Deep sequencing of chromatin immunoprecipitated from the mouse heart using antibodies that recognize EZH2 show direct interaction with genes implicated in cardiac disease (**Table 2**).[52] Interestingly, EZH2 appears to bind novel bi-directional promoter (bdP) sequence to regulate sense and antisense RNA expression. For example, the heart displays altered expression of tumor suppressor related genes *CDKN2B*, *CDKN2A*, and *ARF* encoding the *INK4/ARF* locus at chromosome 9p21 in EZH2-null mice.[10,52] The *ANRIL* antisense is thought to regulate these genes by PcG-dependent silencing.[53] But, whether *ANRIL* directly regulates EZH2 chromatin interaction at the 9p21 region in cardiomyocyte cells remains to be determined. In favor of a role in cardiac homeostasis, individuals homozygous for the SNP allele at the 9p21 region show altered *ANRIL* expression and increased susceptibility to atherogenic plaque development and coronary heart disease (CHD) as well as diabetes.[54,55] While *CDKN2A* expression levels were reduced in 9p21 knockout hearts, there was no evidence for cardiac hypertrophy or cardiovascular pathology.[56] Other studies also report *ANRIL* interactions with PcG proteins such as CBX7 and SUZ12 to regulate *CDKN2B* and *CDKN2A* gene expression.[57,58] Overexpression of *ANRIL* in cultured cells significantly altered the expression of a large number of distant genes proposing *ANRIL* as a *trans* regulatory element.[58] Ontology analysis has identified genes involved in the regulation of chromatin structure and function.[58]

Cardiac hypertrophy and heart failure are associated with changes in the expression of α- and β-*MHC* mRNAs and this shift in myosin-isoform distribution serves important roles in cardiac muscle fiber shortening.[59] The silencing of α-*MHC* in the failing hearts has led renewed interest to restore expression of this gene in hypertrophic tissue.[59] The *MHC* genes are clustered on chromosome 14 in humans and mice (chromosome 15 in rat) and the α- and β- *MHC* genes are separated by an intergenic sequence of ~4.5 kb in length (**Fig. 1**).[60] The β-*MHC* gene is upstream of α-*MHC* and both transcribe mature mRNA approximately 7 kb in length.[60] The complexity of *MHC* gene regulation presents interesting conceptual problems as well as experimental challenges, with the identification of transcripts on opposing DNA strands. This complementary sequence to the canonical mRNA represents the antisense or non-coding RNA.[61] The intergenic region of *MHC* is thought to contain a bdP that transcribes both *AS* β-*MHC* and α-*MHC* in opposite directions.[61] Transcription of *AS* β-*MHC* progresses in the direction of the β-*MHC* gene and is thought to regulate the expression of *MHC* genes in response to pressure overload.[61,62]

The regulation of *MHC* isoforms involves the coordinated actions of core machinery that include DNA-bound transcription factors, chromatin remodeling, and expression of antisense RNA transcripts. Perhaps the most interesting of recent experimental results highlights the complex regulation of the *MHC* genes includes both transcriptional and post-transcriptional changes. Recent experiments in EZH2 mutant mice reveal changes to *MHC* isoform regulation characteristic of the hypertrophic heart.[10] In addition to H3K27me3 modification by EZH2 is the direct involvement of histone-modifying enzymes such as HDAC9, ASXL2, and chromatin remodeling enzymes, such as BRG1 and PARP1 which interact with the bdP (**Fig. 1**).[11,63] DNase hypersensitive sites are also associated with *MHC* gene expression at various developmental stages of the heart.[64] Whatever the role of EZH2, showing its involvement in chromatin dependent association with ncRNA is the first step in revealing how *MHC* isoform expression is regulated in heart disease.

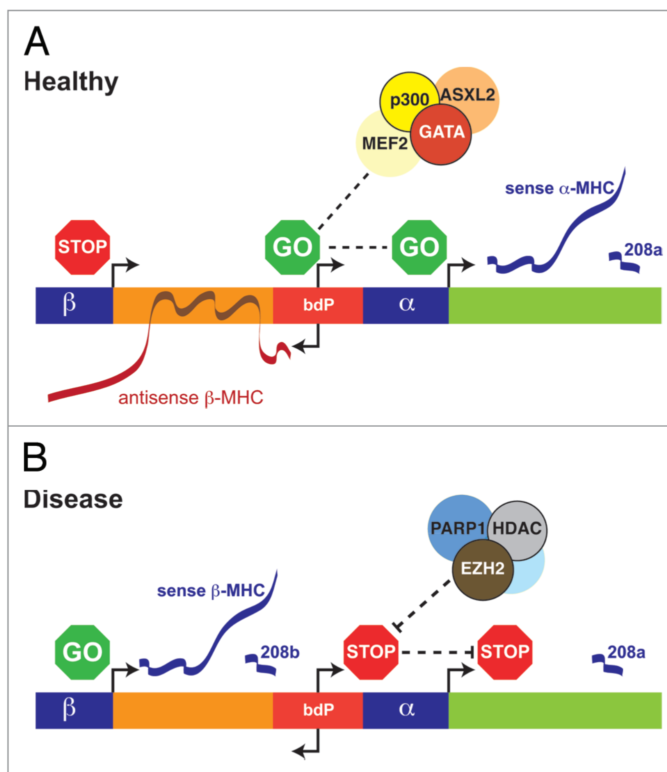

Figure 1. Interplay of chromatin modifications and non-coding RNAs regulate MHC genes in the heart. The expression of cardiac α- and β-*MHC* genes is regulated in (**A**) healthy and (**B**) diseased heart. The bi-directional promoter (bdP) of the α- and β-*MHC* intergenic region comprises binding sequences for GATA, CTF1/NF1, RAR, T3R, MEF-2 transcription factors. Both the α- and β-*MHC* genes encode *miRNA-208a* and *miRNA-208b* that function in heart health and disease. The bdP is known to transcribe *AS* β-*MHC* which serves to regulate β-*MHC* sense (mRNA) transcription by chromatin interaction. The co-regulatory chromatin determinants BRG1, histone deacetylases (HDACs), and EZH2 are involved in the suppression of *AS* β-*MHC* and α-*MHC* genes in disease.

Novel ncRNAs in the Heart

Long ncRNA expression recently described in the heart with regulatory roles involving chromatin modification and function is summarized in **Table 3**. RNA sequencing of the myocardium has revealed specific transcriptome profiles for coding and non-coding transcripts that distinguish the stages of the failing heart.[65] Recent studies have identified more than 1300 previously unannotated exons with altered expression levels in animal models of heart failure.[65] Among these, almost 682 exons displayed

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Table 3. Long ncRNA expression in the heart

Long ncRNA	Cardiac function	Disease association	Expression in disease (↑/↓)	Methods of identification	Mechanism of regulation	Splice variants
ANRIL	Regulation of INK4/ARF locus, genes involved in nuclear and chromatin architecture[56]	Cardiac hypertrophy, atherosclerosis	↑	RNA-ChIP, RACE-PCR, circRNA assays	Chromatin interaction	Reported
cTnI-AS	Regulation of cTnI mRNA[72]	Unknown	Unknown	RACE	RNA duplex formation	None reported
NPPA-AS1	Regulation of NPPA mRNA[68]	Unknown	Unknown	RACE	RNA duplex formation	Reported
AS-UCN	Regulation of sense transcription/translation[124]	Unknown	Unknown	RNase Protection Assay	Overlapping sense transcription	None reported
MIAT or Gomafu	Splicing, retinal cell fate specification[125]	Myocardial infarction	↑	Northern blot, RACE	Chromatin interaction/ *Nanog* TF binding	Reported
Fendrr	Cardiac mesoderm formation[17]	Unknown	Unknown	RACE, RNA-ChIP, ISH	Chromatin interaction	None reported
MHM	Cardiomyocyte Proliferation[126]	Cardiac hypertrophy, arrhythmia	Unknown	Northern blot, In Situ hybridization	Chromatin interaction	Reported
H19	Imprinting and *Igf2* regulation[65]	Hypertrophy & heart failure	↑	RNA-ChIP, Strand-specific PCR	Chromatin interaction	Reported
91H (AS-H19)	Regulation of *Igf2*[127]	Unknown	Unknown	Strand-specific PCR	Unknown	None reported
Kcnq1ot1	Embryonic heart formation, regulation of Cdkn1c, KvLQT1 genes[91]	Unknown	Unknown	RACE, FISH, RNA-ChIP	Chromatin interaction	Reported
FMR1-AS1 or FMR4	Cell proliferation[128]	Proposed	Unknown	RACE, Northern blot	Chromatin interaction proposed	Reported
Air	Embryonic heart formation, imprinting of *Igf2r* in adult hearts[129]	Unknown	Unknown	RNA-ChIP, FISH	Chromatin interaction	Reported
MLC-ALC-1 antisense	Regulation of MLC-1 mRNA[130]	ToF, HOCM	↑	Strand-specific PCR	Unknown	None reported
AS-TGFβ3	Hear chamber formation[131]	Unknown	Unknown	RNase protection assay	RISC-mediated silencing proposed	None reported
sONE (AS-eNOS)	eNOS synthesis[132]	Unknown	Unknown	Strand-specific PCR, In Situ hybridization	Unknown	None reported
SRA	Myogenesis, SRA proteins synthesis[133]	DCM	↓	Strand-specific PCR, Splice variant assays, RNA-ChIP	Chromatin interaction	Reported
AS β-MHC	*β-MHC* gene transcription[61]	Cardiac hypertrophy	↓	Strand-specific PCR	Chromatin interaction	None reported
Braveheart	Cardiovascular lineage commitment[16]	Unknown	Unknown	RACE, native RNA-IP	Chromatin interaction	Reported

ANRIL, antisense non-coding RNA in the INK4 locus; cTnI, cardiac troponin I; NPPA-AS1, natriuretic peptide precursor A-antisense transcript 1; AS-UCN, Urocortin antisense; MIAT, myocardial Infarction associated transcript; MHM, male hypermethylated; MLC-ALC-1, myosin light chain-atrial light chain-1;AS-TGFβ3, transforming growth factor β-3 antisense RNA; SRA, steroid receptor RNA activator; ToF, tetrology of fallot; HOCM, hypertrophic obstructive cardiomyopathy; DCM, dilated cardiomyopathy; RACE, rapid amplification of cDNA ends; FISH, fluorescent in situ hybridization.

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differential expression and the majority (81%) of unannotated RNAs expressed were non-coding RNAs. For example, the expression of *H19* lncRNA was highest in heart failure tissue when compared to cardiac hypertrophy. The function of *H19* in the myocardium remains poorly characterized, as for human heart explants, transcriptome profiling has shown the expression of putative ncRNAs associated with the development of cardiomyopathy.[66] These studies suggest that a large number of novel transcripts are dynamically expressed in the myocardium. Serial analysis of gene expression (SAGE) of different human tissue types has identified cardiac-specific expression of *NCRNA00116*.[67] Despite the tremendous advances in technology used to identify novel RNA species, the physiological function of these molecules remains largely uncharted.[68]

Analysis of ncRNA Dependent-Chromatin Interactions

Recent methodological developments in transcript analysis have seen a tremendous amount of information generated from massive parallel sequencing. While historically difficult to ascribe function to the large number of non-coding RNAs, these transcripts are readily identifiable using RNA sequencing approaches. A number of lncRNAs contain chromatin binding domains and other sequences involved in the interactions with proteins as well as regulating gene expression.[41,43] In the next section, we discuss some of the methodological developments that have enabled the characterization of long ncRNA dependent-chromatin interactions.

Methods Used in the Detection and Characterization of lncRNAs

Important protein-coding genes including those implicated in heart disease have antisense transcription and ncRNA expression.[69,70] Conventionally, in first-strand synthesis, complementary DNA (cDNA) is generated at low temperatures (37 °C) using random/oligo-dT primers that are non-specific to gene sequences as well as lacking strand-specific (5′ to 3′ orientation) information. To distinguish sense from antisense, strand-specific oligonucleotides are used to anneal either mRNA (sense) or ncRNA (antisense) at high temperatures (50–60 °C) followed by first-strand cDNA synthesis. For example, strand-specific primers to cardiac *MHC* and *troponin* genes have been used to quantitatively assay sense (mRNA) and antisense (ncRNA) expression in the heart.[71,72] Recently, several novel procedures have been developed to quantify strand-specific expression of the transcriptome (**Table 4**).[73,74]

Almost 90% of the human transcriptome is alternatively spliced in terminally differentiated cardiomyocytes and neurons.[75] RNA splice variants greatly increase biodiversity of proteins.[76] For example, distinct alternative splicing of the cardiac steroid receptor activator (SRA) transcript can generate SRA protein-coding transcript as well as non-coding regulatory SRA transcript.[77] Consistent with this idea, splice variants in the heart are known to exist for *ANRIL* and regulate circularization of this transcript, whereby one variant type interacts with EZH2 whilst the other is masked for the EZH2 binding domain.[78] Alternative splicing of ncRNA is perhaps key to understanding ncRNA dependent-chromatin interactions. Several strategies, such as exon-scanning and rapid amplification of cDNA ends (RACE) have successfully identified splice variants to cardiac *troponin I-* and *NPPA-* antisense transcripts (**Table 4**).[68,79] Other examples of lncRNAs identified include *KCNQ1OT1* and *HOTTIP*.[50,80]

RNA sequencing (RNA-Seq) approaches generate millions of reads that often fail to accurately identify gene structure as well as result in missing detection of low-abundant transcripts and non-polyadenylated ncRNAs.[81] Transcript profiling can be studied using tiling arrays or targeted RNA CaptureSeq (RNA capture sequencing).[82,83] For example, Mercer et al.[82] used this approach because rare transcripts are thought to occur below the detection limits of conventional RNA-Seq. Surprisingly, the study reported complex ncRNA transcription and widespread expression of novel transcripts.[82] The authors characterize alternative splice junctions to the *HOTAIR* transcript predicted to interfere with PcG binding.[82] Taken together, these data suggest that post-transcriptional splicing can regulate ncRNA dependent-chromatin binding.

Protein expression may also be determined by RNA stability and recent experimental observations suggest dynamic regulation of ncRNA stability in response to specific environmental cues. Pulse labeling of RNA followed by sequencing or 5′-bromo-uridine immunoprecipitation chase—deep sequencing analysis (BRIC-Seq) has identified novel and highly stable lncRNAs.[32] This technique is used to study RNA decay and has revealed that some lncRNAs in fact have short half-lives ($t_{1/2} < 4$ h) such as the cardiac *ANRIL* transcript, *HOTAIR*, *TUG1,* and *GAS5*. Other intriguing observations from the study highlighted that hundreds of short-lived regulatory RNAs designated as short-lived non-coding transcripts (SLiTs) have putative roles in nuclear function.[32] An alternative method of studying RNA stability is transcriptional inhibition by Actinomycin D (ActD).[84] Mouse neuroblastoma cells exposed to ActD over a 32 h period identified over 800 lncRNAs and 12 000 mRNAs that were classified highly stable with a half-life > 16 h or low stability with a half-life < 2 h.[85] The regulatory RNA, *NEAT1* was identified as one of the least stable ncRNAs which is thought to be dynamically regulated. Similarly, global run-on sequencing (GRO-Seq) and native elongating transcript sequencing (NET-Seq) techniques have been be used to assay nascent RNA transcripts.[86,87] These studies identified immediate transcriptional response to estrogen signaling demonstrating that lncRNAs are dynamically regulated.[86] The most obvious conclusion is that low stability lncRNAs are non-functional, but this argument is perhaps overly simplistic, when interpreted slightly differently, long non-coding RNAs may act immediately after transcription to mediate chromatin-dependent interactions.

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Table 4. Methodologies for the detection, characterization and structural analysis of lncRNA. ncRNA-chromatin interaction assays are highlighted with grey background

Method	Advantage
Strand-specific qRT-PCR	Sense and antisense RNA quantification[71,72]
ASSAGE	Reveals transcript direction[73]
RNA ligation using distinct adaptors	Reveals transcript direction[74]
NET-Seq	Transcriptional pausing[87]
GRO-Seq	Immediate, transient changes to transcriptome[86]
Exon-scanning	Splice variant detection[68,79]
RACE	Splice variant detection, Obtain full-length transcript sequence[50,80]
RNA CaptureSeq	Detection of transcripts of low abundance, Novel splice variant detection[82]
BRIC-Seq	Transcript stability, RNA decay[32]
SAGE (SuperSAGE)	Novel, tissue-specific lncRNA detection[67]
$PolyA^-$ RNA-Seq	Identification of bimorphic transcripts and circular RNAs[104]
RNA bisulfite conversion	RNA methylation, RNA folding, footprint sequences[73]
PTES identification	Splice variants, circular RNA prediction[109]
FragSeq	Intra- and inter- RNA base pairing[112]
RNaseR assay	Circular transcriptome studies[104,107]
Native chromatin preparation	Purifies CARs, $PolyA^-$ ncRNAs[134]
RNA-FISH	Cellular compartmentalization of transcripts, chromatin interaction[89,90]
RNA-ChIP	Protein-dependent RNA interaction with chromatin[80]
Native RNA-ChIP	Protein-dependent RNA interaction with chromatin[92]
ChIRP	RNA-dependent chromatin interaction[95]
CHART	RNA-dependent chromatin interaction[96]
HITS-CLIP	Cross-linking of directly interacting RNA-protein complexes[97]
PAR-CLIP	Cross-linking of directly interacting RNA-protein complexes[98]

ASSAGE, asymmetric strand specific analysis of gene expression; GRO-Seq, global run-on sequencing; NET-Seq, native elongating transcript sequencing; RACE, rapid amplification of cDNA ends; BRIC-Seq, 5′-bromo-uridine Immunoprecipitation chase-deep sequencing; SAGE, serial analysis of gene expression; PTES, post-transcriptional exon scrambling; CARs, chromatin associated RNAs; FISH, fluorescent in situ Hybridization; ChIP, chromatin immunoprecipitation; ChIRP, chromatin Isolation by RNA purification; CHART, capture hybridization analysis of RNA targets; HITS-CLIP, high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation; PAR-CLIP, photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation.

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Long ncRNA-Chromatin Interaction Assays

Long ncRNAs that stably interact with chromatin at specific genomic sites can be detected by fluorescent in situ hybridization (FISH) of the target RNA using antisense probes.[88] FISH has traditionally been the method of choice to study long ncRNA dependent-chromatin interactions.[89,90] More recently, FISH was employed to assay changes in chromatin architecture for *KCNQ1OT1* a lncRNA that regulates *KCNQ1* expression in the developing heart.[91] Alternatively, locus-specific lncRNA interactions can be examined using formaldehyde fixation and chromatin immunoprecipitation methods (RNA-ChIP) that use antibodies that recognize RNA-binding proteins such as EZH2 and G9A.[80] Alternatively, native RNA-ChIP using MNase digestion have also been successfully applied to the study of chromatin associated RNAs.[92] In striking contrast

to formaldehyde crosslinking, immunoprecipitation of native soluble chromatin allows for direct mapping of mono-, di- and tri-nucleosomal structures.[93]

Long ncRNAs can interact in a locus-specific manner using homologous complementary sequences.[94] The applicability of biotinylated RNA tiling probes complementary to target lncRNA was recently used to immunoprecipitate interacting DNA sequences and proteins. Examples of these methods include ChIRP (chromatin isolation by RNA purification) and CHART (chromatin hybridization analysis of RNA targets) which have identified novel genome-wide interactions for *HOTAIR* and *ROX2*.[95,96] In fact, with the advent of high-throughput sequencing it has been possible to identify novel RNAs using crosslinking immunoprecipitation (HITS-CLIP) and photoactivatable ribonucleoside enhanced crosslinking and immunoprecipitation (PAR-CLIP).[97,98] These methodologies were recently used to identify the interaction of EZH2 with several ncRNAs, including *ANRIL* which has been associated with many diseases including coronary artery disease, diabetes and cancer.[99]

Structural Analysis of lncRNAs

Besides sequence-based chromatin recognition, RNA folding can also influence ncRNA dependent-chromatin interactions.[100] For example, genes that code for *DMD*, *P450*, *MLL,* and *ETS-1* produce circular transcripts with diverse functions.[101-104] The hypertrophy responsive *NCX1* gene is thought to produce circular poly(A-) transcripts in the human heart, however the biological significance of circular RNAs has remained elusive.[105] Recent evidence now suggests that circular RNAs function as miRNA sponges that compete with RNA binding proteins to form a class of post-transcriptional regulators.[106,107] Accordingly, circular antisense RNAs are targeted by RISC components for gene regulation.[108] The mechanism of RNA circularization is a result of non-canonical post-transcriptional exon scrambling (PTES). Non-canonical PTES appears to be a predominant event in human liver as well as the heart.[109] Because of their low abundance, the majority of circular transcripts are largely undetectable by conventional RNA-sequencing. To investigate the circular component of the transcriptome, protocols employ RNaseR, an enzyme that degrades linear but not circular transcripts.[104] Coupled with RNaseR, next generation sequencing has identified PTES mediated circular RNA transcripts to hundreds of human genes, the majority of which were not polyadenylated.[104] In fact, circular and linear forms of cardiac antisense RNA, *ANRIL* have been reported.[78] The expression of circular *ANRIL* might be associated with atherosclerotic vascular disease. Thousands of human mRNA and ncRNA transcripts are extensively methylated[110] and these RNA modifications are thought to alter Argonaute binding as well as transcript folding.[111] Moreover, recent identification of specific ncRNA structures such as the TINCR boxes regulate the interaction of these transcripts with regulatory proteins.[100] FragSeq or fragmentation sequencing is a novel method that integrates RNA structure analysis with genome-wide sequencing.[112] The Nuclease P1 enzyme is used to cleave single-stranded nucleic acids thereby preserving the intra- and inter-molecular RNA interactions. The development of these methodologies has revolutionized genome-wide analysis of cellular RNAs, which will be critical in defining regulatory networks at the genomic scale.[113]

Conclusions and Future Considerations

Recent experimental observations show lncRNAs regulate cardiac gene expression. This is probably best exemplified at the bidirectional promoter of the *MHC* genes which involves the interaction of EZH2 with the antisense β-*MHC* transcript to regulate *MHC* isoform shift (**Fig. 1**). While always considered to be integral elements in the post-transcriptional control of gene expression it is the recent technological developments that have been critical to understand the role of ncRNAs in the heart. The advent of massive parallel sequencing has brought improved understanding of the regulatory mechanisms underlying cardiac pathology and developmental growth as well as integrating functional genomics. Although the relevance of the non-coding genome to cardiac disease has mainly been studied in the context of the widespread disruption of expression, studies now show that ncRNAs are also critical determinants of gene regulation. Taken together with their emerging role with chromatin modification, the non-coding genome should provide new strategies and specific targets to prevent, restore or reverse the effects of pathological hypertrophy in the failing heart.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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