Trends in Cardiovascular Medicine ▮ (2015) ▮▮▮–▮▮▮

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The promise of enhancer-associated long noncoding RNAs in cardiac regeneration

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ABSTRACT

Heart failure is a worldwide epidemic and represents a major cause of morbidity and mortality. Current clinical therapies for heart disease prolong survival by protecting the viable muscle, but they are unable to replenish lost cardiomyocytes to restore function. Over the last decade, the notion of promoting cardiac regeneration has engendered considerable research interest. New strategies envisage the transfer of stem cells into the damaged myocardium, the mobilization of cardiac precursor cells, the promotion of cardiomyocyte proliferation in situ and direct reprogramming of non-cardiac cells into electromechanically coupled cardiomyocytes. The molecular and cellular mechanisms underpinning these different regenerative avenues are under the control of integrated transcriptional programs, which are ultimately dependent on epigenomic reprogramming and reorganization of the genome nuclear architecture. Today, it is becoming evident that regulatory noncoding RNAs play fundamental roles in all these aspects of gene regulatory network activity. In particular, thousands of long noncoding RNAs are dynamically expressed across the entire genome during lineage-specific commitment, specialization, and differentiation, as well as during the response to environmental cues. Here, we review this emerging landscape, focusing particularly on a unique class of lncRNA emerging from enhancer sequences, the enhancer-associated lncRNAs, in the context of cardiac regeneration. We propose that characterizing and manipulating these enhancer-associated transcripts could provide a novel approach to awaken the dormant regenerative potential of the adult mammalian heart. Ultimately, this could lead to targeted noncoding RNA-based enhancer therapies to improve effectiveness of current regenerative strategies and provide new avenues for repair.

Introduction

Coronary artery disease is the most frequent cardiovascular disorder and leads to acute myocardial infarction. Myocardial infarction frequently progresses into maladaptive cardiac remodeling and congestive heart failure, which therefore affects millions of people worldwide and is a major cause of morbidity and mortality [1]. Despite several efforts to improve treatments during the acute phase of myocardial infarction, the WHO estimates that rising life expectancy coupled with adverse trends in cardiovascular risk factors, including obesity and type II diabetes, might lead to a doubling of the incidence of cardiovascular disease by 2050. Currently, transplantation remains the only therapeutic option for end-stage heart failure. However, the lack of organ donors limits the access to transplantation to a small number of patients each year. It is therefore urgent to develop alternative strategies to treat heart failure patients. In this context, induction of cardiac regeneration in the damaged heart could represent an attractive therapeutic approach [2,3]. This will only be possible if the pathways and underlying molecular mechanisms important for inducing repair are identified.

Research in the authors' laboratory is supported in part by grants from the Swiss National Science Foundation, Switzerland (T.P.; Grants nos. 31003A-127590 and 406340-128129).

The authors have indicated that there are no conflicts of interest.

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http://dx.doi.org/10.1016/j.tcm.2015.01.014

During the past decade, regenerative medicine for heart disease has engendered a lot of attention. From the old dogma, which assumed that the postnatal heart had no capacity to generate new cardiomyocytes, we have gradually shifted to a general acceptance that the heart demonstrates some ability for self-renewal. However, the rate of cardiomyocyte production in the adult heart is extremely low [4]. In addition, the damaged heart has poor regenerative potential and heart failure develops following injury [5]. Nevertheless, these findings created great expectations in the community. Indeed, if one assumes that the heart possesses the necessary elements for regeneration, strategies can be envisaged to reinforce basic cellular and molecular mechanisms and tip the balance toward more regenerative repair [3]. There are principally two strategies to induce regeneration in the adult heart. First of all, precursors of cardiomyocytes can be transferred into the damaged heart, hoping that the cardiac environment provides the indispensable factors to eventually promote engraftment, terminal differentiation into mature cardiomyocytes, and electro-mechanical coupling to the host tissue (Fig. 1A). The main challenge in cell therapies for heart disease is to identify a suitable source of cardiac precursors cells (CPC). Therefore, several different cell types have been evaluated. Embryonic stem (ES) cells and induced pluripotent stem (iPS) cells are unlimited sources of precursors [2]. In particular, the production of iPS cells represents a tremendous opportunity for developing patient-specific cell therapies [6]. However, specification and differentiation must be tightly controlled in order to not produce unwanted cell types in the heart and cause major adverse effects. On the other hand, adult stem cells also represent suitable sources of precursors. Ideally, these cells should be isolated from the heart. So-called cardiac stem cells and CPC have indeed been identified in the myocardium, but these cells are rare and their exact nature remains to be completely established [7]. In addition, their real potential in cell replacement therapies for heart failure still needs to be demonstrated. Nevertheless, direct isolation of autologous CPC from the adult human heart would considerably reduce the problems associated with immune rejection following transfer. The second basic strategy consists in activating regenerative pathways in the heart (Fig. 1B). Resident CPC could be mobilized, induced to proliferate, and stimulated to differentiate into mature cardiomyocytes directly in situ. Inspiration comes from pathways governing cardiomorphogenesis in the developing heart [8]. In addition to candidate approaches targeting developmental pathways, more unbiased methods have also been used to identify mechanisms that are able to force cardiomyocytes to reenter the cell cycle [9]. Proliferation of dedifferentiated cardiomyocytes represents the basis of regeneration in species like the Zebrafish or in the neonatal mammalian heart [10–12]. Finally, researchers have recently used induced reprogramming to convert differentiated cells of the heart, specifically cardiac fibroblasts, directly into cardiomyocytes [13]. Using this approach, one can bypass iPS cell production to directly obtain the cell type of interest. Moreover, direct reprogramming can also be demonstrated following induction in vivo, within the heart [14,15]. Direct reprogramming of somatic cells into cardiomyocytes certainly holds great promise in cardiac regenerative medicine and justifies further investigation.

Although the current regenerative strategies have distinct characteristics linked to proliferation, differentiation, and reprogramming, they all fundamentally depend on overlapping cellular and molecular processes [16]. The gene regulatory networks that govern these processes are under the control of the integrated activity of core cardiac lineage, specifying transcription factors including Mesp1, Nkx2.5, Mef2c, Gata4, Tbx5, and Hand2. These transcription factors interact in a combinatorial and self-reinforcing manner at target cis-regulatory modules to elicit specific temporal and spatial gene programs [16–18]. Coordinated binding of the core transcription factors is coupled with dynamic remodeling of the underlying chromatin, leading to global epigenomic reprogramming and reorganization of the genome nuclear architecture [19]. These genomic processes dictate protein-coding gene expression that is ultimately responsible for cellular fate, phenotype, and behavior. Importantly, the regulation of the proteome, the transcriptome, the epigenome, and the nuclear architecture is highly integrated to coordinate outputs of otherwise disparate molecular networks. Within this context, it has recently emerged that the noncoding portion of the genome generates a vast repertoire of noncoding RNAs with regulatory function on cell-specific gene networks [20,21]. An important subclass of these transcripts is derived from enhancer sequences [22,23]. This exciting discovery opens the new era of enhancer therapy to treat diseases by modulation of enhancer-associated noncoding RNAs and subsequently their target transcriptional programs.

The regulatory role of the noncoding genome

The analysis and interpretation of gene regulatory network activity have traditionally been protein-centric. However, recent high-throughput sequencing technologies have begun to illuminate our understanding of the human genome. Specifically, only 1–2% of the genome appears to code for proteins. The remaining 98% represents the noncoding portion of the genome [20,21]. This genomic “dark matter” is dynamically transcribed, producing thousands of RNAs with no protein-coding potential, globally named noncoding RNAs (ncRNAs). Emerging evidence indicates that these noncoding transcripts are responsible for complexity in gene regulation, which underpins specialized biological processes during development and in adulthood [24]. The vast majority of the 20,000 human proteins are similar in number and orthologous in function to those found in distantly related species. In contrast, the number of noncoding genes is proportional to the developmental complexity among animals. Nevertheless, an increasing number of studies are demonstrating that these structurally diverse ncRNAs control every aspect of gene regulatory network activity, including transcriptional control, post-transcriptional gene regulation, epigenetic targeting, and nuclear genome organization [25]. In this context, the roles of ncRNAs in cardiac development, disease, and ultimately regeneration remain to be defined. However, incorporating ncRNAs within the logic governing cardiac gene regulatory networks provides unprecedented opportunities for therapeutic intervention and may facilitate the promotion

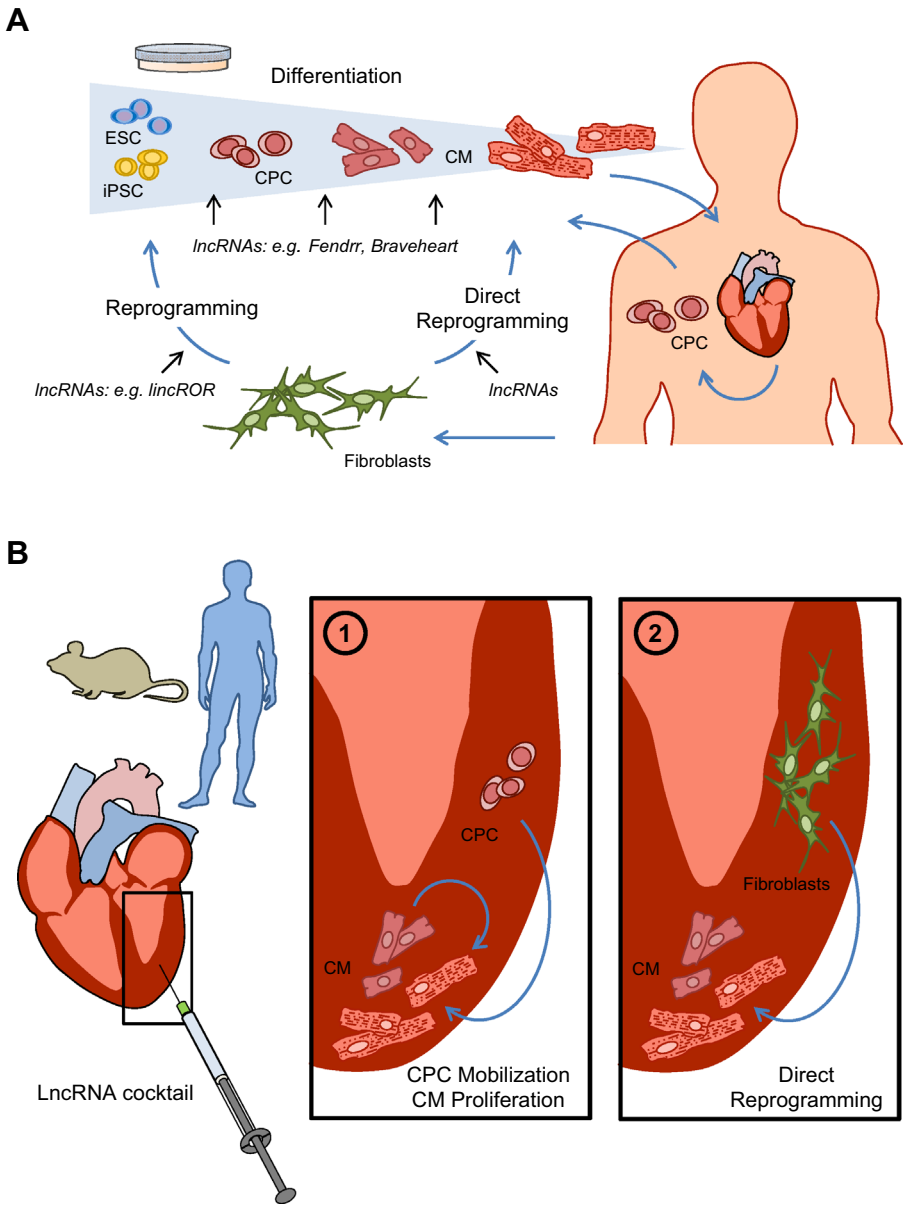

Fig. 1 – Current avenues for inducing regeneration in the damaged heart. The putative functional implication of lncRNAs is indicated by arrows. (A) Cell replacement therapies for heart disease rely on the identification of appropriate sources of cardiac precursor cells (CPC). These cells can be isolated from the heart or derived from pluripotent stem cells following controlled differentiation in vitro. Embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC) represent two types of pluripotent stem cells. Somatic cells such as fibroblasts can be reprogrammed to give rise to iPSC. In addition, fibroblasts can also be directly reprogrammed into cardiomyocytes. These different cell types can all be transferred back into the heart for potentially replenishing the cardiomyocyte pool lost after injury. (B) Cardiac regeneration can also be induced by activating appropriate regenerative pathways within the heart. The target populations in this case are CPC, cardiomyocytes, or cardiac fibroblasts. Mobilization of CPC and induction of cardiogenic differentiation are usually achieved by reactivation of developmental pathways in the adult heart (1). In another approach, cardiomyocytes can be induced to reenter the cell cycle following partial dedifferentiation (1). Finally, direct reprogramming of cardiac fibroblasts represents another way to produce new cardiomyocytes in the injured heart following intramyocardial transfer of inducing factors (2).

of a regenerative response in the heart. The aim of this short review is therefore to provide insights into the roles of long noncoding RNAs (lncRNAs) within the cardiac gene regulatory network and their potential in cardiac regenerative therapies.

The RNAs produced by the noncoding genome are rich and diverse in biogenesis, structure, and function [25]. They are currently parsed based on size, with small regulatory ncRNAs defined as those that are less than 200 nucleotides in length and long ncRNAs (lncRNAs) that are more than 200 nucleotides in length [26]. Many of the originally identified ncRNAs, including transfer, ribosomal, small nuclear, and small nucleolar RNAs have well-established roles as structural and functional components of the splicing and translational machineries. Recently, much interest has arisen in a

heterogeneous class of small regulatory ncRNAs that can directly affect the function and expression of protein-coding genes. These small transcripts include PIWI-interacting ncRNAs, endogenous small interfering RNAs, and microRNAs (miRNAs). With relevance to cardiac regeneration, miRNAs represent the most extensively studied class of small regulatory ncRNAs [27]. These 22–23 nucleotide single-stranded RNAs guide RNA-induced silencing complexes to their target messenger RNAs (mRNAs). Recognition involves partial sequence complementarity, primarily with the target gene 3′UTR. Indeed, miRNAs are predicted to mediate fine-tuning of coding gene expression via post-transcriptional gene silencing of up to 60% of mammalian mRNAs. Not surprisingly, miRNAs have been shown to play critical roles during cardiac development, pathological remodeling, and cardiac regeneration [28]. For instance, miRNAs have important regulatory roles in directing cell fate decision during cardiogenesis while their expression is often regulated by important cardiac transcription factors. Interestingly, cardiac biological processes implicated in regeneration are all significantly influenced by miRNA-dependant regulatory networks. These include cardiac specification and differentiation, cardiomyocyte dedifferentiation and proliferation, and direct reprogramming of cardiac fibroblasts into cardiomyocytes. For instance, combinations of muscle-specific miRNAs, namely miR-1, miR-133, miR-208, and miR-499, are sufficient to reprogram mouse fibroblasts into cardiomyocytes in vitro and in vivo, reducing infarct scar size and improving cardiac performance [29,30]. In addition, a number of miRNAs participate in pathophysiological remodeling in the heart. Many miRNAs that are modulated in the stressed myocardium are also implicated in induced cardiogenesis, suggesting that miRNA-mediated regenerative pathways could be activated in cardiac tissues upon damage. This has been the subject of recent reviews [27,28], and we will therefore focus herein on a newly identified class of ncRNAs, namely lncRNAs.

Long noncoding RNAs

LncRNAs comprise the bulk of the noncoding transcriptome [20,21]. They represent a structurally and functionally diverse class of regulatory ncRNAs, which can range up to tens or even hundreds of thousands of nucleotides in size. They may or may not be subject to polyadenylation and alternative splicing. LncRNAs can be nuclear or cytoplasmic, although the most investigated to date are generally enriched in the nucleus. Their expression in particular subcellular compartments is probably related to specific function associated with particular lncRNAs. Classification of lncRNAs is currently quite primitive and is primarily based on their genomic location. For example, long intergenic noncoding RNAs are located between coding genes within intergenic space, not overlapping exons of other coding genes. LncRNAs can also reside within introns of coding genes occasionally overlapping with and sharing exons. An early characterized class includes the so-called natural antisense transcripts, which are produced from the opposite strand of a coding gene. Their transcription initiation site is typically downstream relative to that of the coding gene. In addition to their geographical diversity, lncRNAs are highly versatile macromolecules that can pair with other RNA templates or with DNA to form triplex structures [25]. These molecules can also interact with a vast repertoire of proteins, highlighting their tremendous regulatory potential. A number of examples from different fields of science allowed the delineation of specific modes of lncRNA action. In particular, lncRNAs can act as molecular signals to target specific elements in both the transcriptome and the genome [24]. They also can act as specific molecular scaffolds for protein–protein interactions and have roles as molecular decoys for both nucleic acids and proteins [25].

Having said this, lncRNAs are emerging primarily as important regulators of gene expression at the transcriptional and post-transcriptional level. They demonstrate distinctive roles in modulating tissue-specific epigenomic states and nuclear organization, which are critical for correct gene regulatory network activity [24]. Several characteristics of lncRNAs make them ideal molecules to provide the nucleus with a catalog of molecular "address codes" to guide epigenetic and transcriptional regulatory events. In particular, lncRNAs can rapidly and efficiently operate both in cis, at their site of transcription, and in trans, at remote locations in the genome (Fig. 2). For instance, a subclass of lncRNAs associated with active enhancers has recently been found to activate neighboring genes in cis using mechanisms involving chromatin looping between the enhancer sequences and their target gene (see below). However, many characterized lncRNAs act primarily in trans as decoys or recruiters for transcription factors and chromatin-remodeling complexes to activate or silence specific expression programs [24]. LncRNAs have particular affinity with RNA-binding proteins such as components of the Trithorax and Polycomb complexes. In addition, other trans-regulatory roles have also been identified. Some lncRNAs have a significant impact on mRNA degradation, translation, or splicing by binding to proteins or components of ribonucleoprotein complexes [25]. Finally, some lncRNAs have been reported to function as miRNA sponges, targeting these small regulatory ncRNAs away from their target mRNAs [31]. This establishes so-called competitive endogenous networks, in which expression of each participant is closely dependent on expression of the two other members.

One unique characteristic associated with lncRNAs is that they exhibit richer tissue specificity when compared to coding genes and small regulatory ncRNAs [25]. This is in particular the case in the heart [32]. This suggests that one primary function of lncRNAs during cell specification and differentiation is to modulate combinations of ubiquitously expressed chromatin-modifying complexes in a highly cell-specific manner. In turn, this governs the epigenomic state of target genomic regions and promotes adoption of particular fate. Interestingly, many lncRNAs appear to function during development to repress non-appropriate gene networks through the recruitment of repressive chromatin-modifying complexes at specific sites within the genome [33]. Therefore, specialization appears to occur via lncRNA-mediated restriction of particular states rather than activation of alternative states. Moreover, global reorganization of the epigenome and of the nuclear architecture is crucial for induced reprogramming to a pluripotent state, i.e., production of induced pluripotent stem cells (iPS) [34]. A canonical example is the lncRNA known as "Regulator of Reprogramming" (linc-ROR). Overexpression or depletion of this lncRNAs

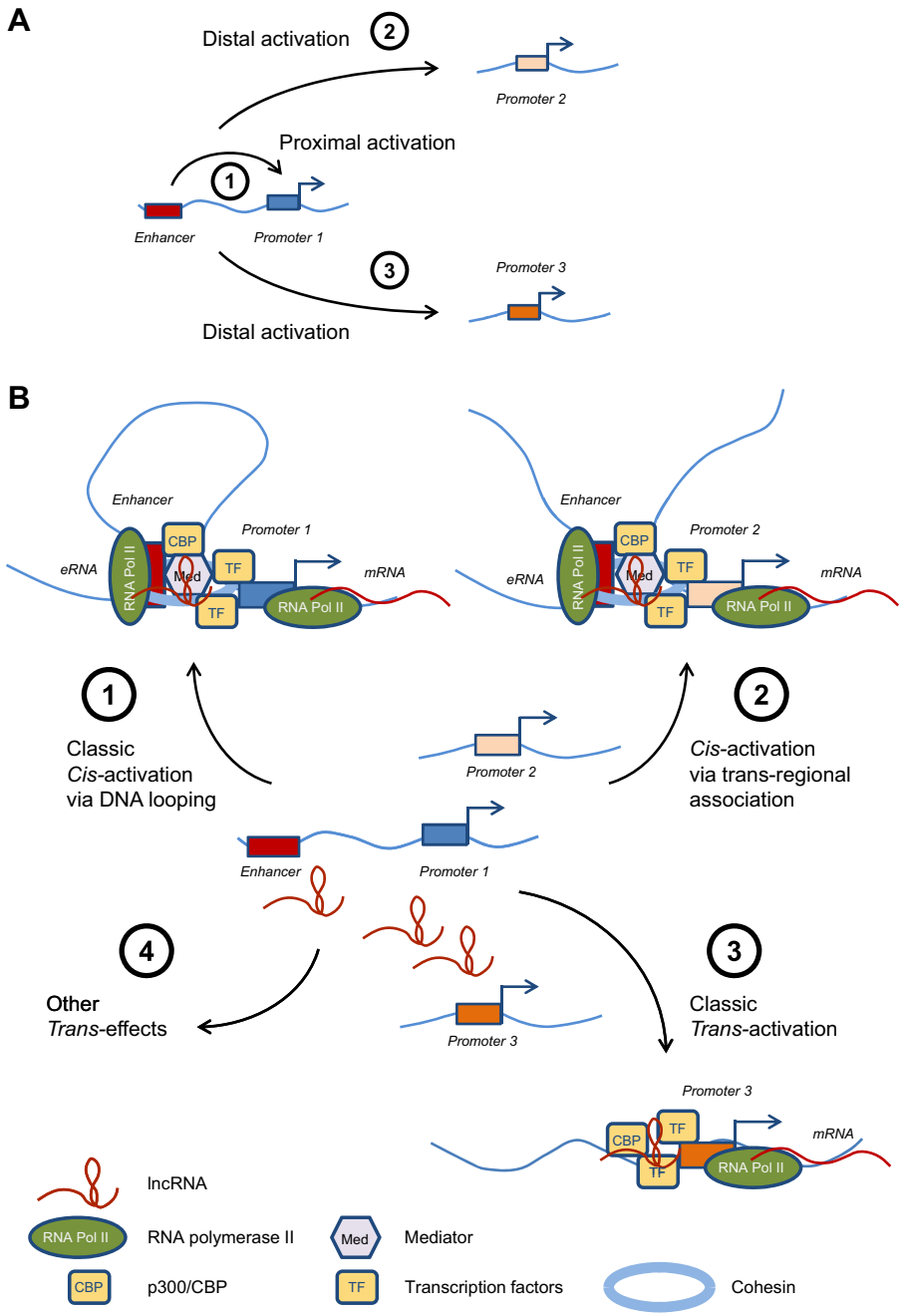

Fig. 2 – Role of enhancer-associated lncRNAs in cis- and trans-regulation of coding gene expression. (A) Enhancers activate proximal (1) and distal genes at remote locations in the genome (2) and (3). (B) Enhancer-associated lncRNAs exert regulatory function on coding gene expression via diverse mechanisms. In classic cis-regulation, a nascent and tethered enhancer-associated lncRNA induces and stabilizes DNA looping between the enhancer and the promoter of the adjacent coding gene (1). In a similar cis-regulatory mechanism, the nascent and tethered enhancer-associated lncRNAs promote association of the enhancer with a promoter positioned at a remote location in the genome (2). In classic trans-regulation, the enhancer-associated lncRNAs are released from their site of transcription and activate remote gene promoters via recruitment of the transcription machinery (3). LncRNAs can have other trans-regulatory functions, e.g., cytoplasmic functions (4).

leads to a higher or lower efficiency of reprogramming fibroblasts to induced pluripotent stem (iPS) cells [35]. The capacity of lncRNAs to control epigenomic remodeling and organization of the nuclear architecture is therefore key for lineage-specific transcriptional regulation during cellular specification, differentiation, and reprogramming, all processed that are central to elaborate a coordinated regenerative response.

Enhancers: Key information processing units within the genome

Tight control of cell proliferation and functional specialization is fundamental for biological processes that are implicated in developmental and adaptive responses in

metazoans. These processes require cells to respond to developmental and environmental cues by executing differential transcriptional programs from a single set of genetic material. Within the gene regulatory networks, enhancers are the primary information processing units that enable specificity of transcriptional gene programs [36]. Enhancers were initially characterized as cis-regulatory DNA elements that activate target genes over large distances in an orientation-independent manner [37]. They contain clusters of binding sites for lineage-determining transcription factors (TFs), which thereby regulate gene expression in a temporal and spatial fashion. Therefore, cardiac-specifying TFs and chromatin regulators coordinate the activation and repression of complex transcriptional networks that underpin cardiac gene regulatory networks. This primarily occurs at enhancer sequences, which selectively recruit TFs and integrate specific chromatin-state transitions to elicit appropriate cellular responses. Current estimates based on the use of high-throughput epigenomic screens suggest that more than 1 million putative enhancers exist in the human genome, vastly outnumbering protein-coding genes. On average, each coding gene is potentially regulated by tens of enhancers. This observation has led many to suggest that the complexity of enhancer utilization is responsible for the highly complex gene expression programs required for specialized developmental programs.

Dynamic regulation of lineage-determining transcriptional programs is particularly evident during cardiac development where complex patterns of gene expression are exquisitely modulated during cardiac morphogenesis [18]. A genome-wide epigenomic screen identified 3000 cardiac enhancers in the developing mouse heart at embryonic day 11.5, based on cardiac-specific enrichment of p300 [38]. Accordingly, disruption of cardiac gene regulatory networks at the level of transcription factors, chromatin-remodeling complexes, and enhancer sequences underpins congenital heart disease and susceptibility to acquired adult heart pathologies [18]. Consistent with these ideas, the epigenome has been recently profiled at four stages during differentiation of mouse embryonic stem cells into cardiomyocytes, recapitulating developmental programs [39]. Through interrogating specific chromatin marks (H3K4me1 and H3K27Ac) to parse poised vs. active enhancers, over 80,000 enhancers were identified across the developmental time course in vitro. Interestingly, the sets of active enhancers were largely unique, even in closely related cellular lineages, demonstrating that rapid stage-specific chromatin-state transitions occur at enhancer regions during cardiac specification, differentiation, and maturation. In addition, some Mendelian diseases have been shown to occur as consequences of disruption of enhancer-binding TF function [40]. Mutations within noncoding DNA elements, including enhancers, also have similar developmental consequences. Recent studies highlight that disruption of enhancer activity by trait-associated single nucleotide polymorphisms (SNPs) is a common phenomenon in complex diseases. For example, recent genome-wide studies profiling enhancers in nine cell types demonstrated that trait-associated SNPs were enriched specifically in enhancers active within biologically relevant cell types [41]. These data suggest that human genetic variation linked to specific traits and disease can contribute to phenotypes by affecting enhancer activity and subsequently gene regulatory networks hardwired by the affected enhancers.

Recently, investigators have identified regions of the genome, where sets of enhancers are clustered together. These enhancers are currently termed “super” or “stretch” enhancers [42,43]. They are characterized by a 10-fold higher median length than regular enhancers, enrichment for lineage-specific TFs, and greater association with chromatin-remodeling factors. Importantly, these super-enhancers produce higher levels of enhancer-associated ncRNAs as compared to canonical enhancers. Furthermore, gene encoding master regulators of cell identity and specialization are located proximally to super-enhancers. Approximately 250 super-enhancers were found to be active within the adult human left ventricle. These were significantly enriched with genetic variants associated with cardiac electrocardiographic traits [41]. Two recent studies used global epigenomic screens to examine cardiac enhancer dynamics during pathological remodeling in the heart. One study assessed distal GATA4-bound enhancer dynamics during adult heart homeostasis [44], while the other assessed the dynamics of enhancer activity, defined by extensive acetylation of histone 3 lysine 27 (H3K27ac), during pressure overload-induced cardiac hypertrophy [45]. Both studies demonstrate that context-specific transcription factor occupancy underlies stage-specific transcriptional events during cardiac homeostasis and disease. Moreover, the epigenetic landscape at enhancers is a key determinant of gene expression reprogramming in cardiac hypertrophy. These findings place enhancers as the central units within the gene regulatory networks that underpin these maladaptive processes. Interestingly, these data are also consistent with reactivation of developmental pathways in the stressed heart. Enhancer remodeling is therefore likely to be the key regulatory determinant for the reactivation of dormant regenerative pathways within the adult mammalian heart.

Enhancer-associated long noncoding RNAs

Enhancers positively regulate the expression of their target genes via remodeling of the local epigenetic landscape and the formation of three-dimensional chromatin loops to facilitate RNA polymerase II (RNAPII) initiation and elongation at target gene transcriptional start sites (Fig. 2) [46]. As discussed above, these critical genomic regulatory processes are under the control of regulatory ncRNAs. Over recent years, evidence has emerged of pervasive RNA transcription at active enhancer sequences during different cellular contexts [22]. Original reports characterized broad patterns of transcription at active enhancers in neurons and T cells. Subsequently, approaches able to detect nascent transcripts confirmed the production of enhancer-associated lncRNAs (elncRNAs) in prostate and breast cancers. These reports demonstrated that RNAPII complexes were enriched at enhancer elements and rapidly responded to signal transduction via elncRNA transcription.

The integration of various genomic technologies, including ChIP-Seq, RNA-Seq, and chromosome conformation capture, has allowed defining the properties of elncRNAs. Enhancer-associated lncRNAs are transcribed from enhancer regions characterized by high monomethylation of histone H3 lysine 4

(H3K4me1) relative to trimethylation (H3K4me3) [47]. The expression of elncRNAs correlates also with the activation of the enhancers as indicated by H3K27Ac [47]. Generally, elncRNAs exhibit similar levels of transcription as compared to mRNAs but exhibit much shorter half-lives. Importantly, developmental and signal-dependent changes in elncRNA expression are highly correlated with expression of target genes, in particular in the heart [47–49]. Thus, elncRNAs are dynamically expressed upon developmental differentiation cues and upon signal transduction events orchestrated by signal-dependant transcription factors or nuclear receptors. Interestingly, elncRNAs exist as two primary transcripts [47]. One is unidirectional, multi-exonic, spliced, and polyadenylated elncRNAs. The other is bidirectionally transcribed and non-polyadenylated. The latter is more common and found at most enhancers. Despite these pieces of evidence, controversy persists as to whether elncRNA production is required for enhancer activity. However, a number of recent studies have demonstrated that using loss-of-function approaches that targeted degradation of elncRNA is sufficient to reduce expression of adjacent coding genes [50,51]. In some instances, depletion of elncRNAs results in reduced enhancer–promoter chromatin looping [52]. Therefore, elncRNAs could play a role in the initiation and stabilization of the loop, which ultimately dictates the integration of enhancers within gene regulatory networks. In contrast, many elncRNAs are not required for the looping process itself. Instead, elncRNAs appear to function once the loop is already formed, to facilitate RNAPII pause release at target transcriptional start sites and to promote transcriptional elongation [53]. However, both mechanisms suggest cis-regulatory function for elncRNAs. Indeed, the main characteristics of elncRNAs, including their low expression, their absence at genomic regions other than their site of transcription, and the minimal effects of loss-of-function on non-adjacent coding genes, are mainly consistent with a predominant cis mechanism of action. Therefore, elncRNAs could exert their function via promoting direct interactions between enhancers and neighboring or distal genomic regions, refined within specific three-dimensional domains (Fig. 2). Nevertheless, this does not rule out the possibility of classic trans-regulatory function for elncRNAs. Classic trans-regulation is suggested by the fact that depletion of certain elncRNAs leads to change in the expression of a greater number of genes than its predicted target gene alone [22]. Considering the roles that elncRNAs have in inducing nuclear architecture reorganization, trans-regulatory function might represent the capacity of elncRNAs to stabilize the genome in topological three-dimensional domains, favoring the adoption of particular cell fate during development and cellular specialization. Controlled remodeling of such nuclear domains might be of major importance to hardwire cardiac gene regulatory networks for reprogramming and induction of a regenerative response in the adult human heart.

Enhancer-associated long noncoding RNAs in cardiac differentiation and in development

LncRNAs are emerging as key regulators for both maintaining stemness in ES and precursor cells and specifying these cells toward the three primary germ layers, i.e., the mesoderm, endoderm, and ectoderm. In a pioneering study, these properties were systematically investigated via a loss-of-function approach in mouse ES cells [54]. Most of the functional lncRNAs were shown to bind diverse chromatin reader, writer, and eraser regulatory protein complexes to control specification and specialization into one of the three germ layers and their derived lineages. In particular, some of the identified lncRNAs determine specification into the mesoderm through repression of non-appropriate cell fates. However, characterization of lncRNAs in cardiac lineage commitment is still in its infancy. Here, we will highlight recently described lncRNAs, specifically those associated with enhancer sequences that have been implicated in cardiac biology.

Despite not being directly associated with a cardiac enhancer, the lncRNA Braveheart (Bvht, AK143260) is a lncRNA transcribed from an important regulatory locus, which has been previously shown to be rich with heart-specific enhancers [55]. Bvht was discovered based on its unique expression pattern during cardiac differentiation in mouse ES cells. However, Bvht is also enriched in the adult heart as compared to other tissues, suggesting that it represents an important lncRNA for cardiac lineage specification and differentiation. Indeed, Bvht loss-of-function in mouse ES cells resulted in perturbed differentiation and reduced formation of cardiomyocytes. Interestingly, Bvht appears to be directly upstream of Mesp1, an essential transcription factor that marks early cardiac precursor cells during development. Mesp1-positive precursor cells have the capacity to generate all cell types of the heart, including endothelial cells, smooth muscle cells, and cardiomyocytes. Therefore, Bvht, via its regulation of Mesp1, is responsible for the correct temporal activation of cardiogenic lineage-determining TFs such as Nkx2-5, Hand1, Hand2, Tbx2, Gata6, and Gata4. Through this critical modulation of the cardiac-specifying gene regulatory network, Bhvt could be necessary for the lineage transition from nascent to cardiac mesoderm and the subsequent differentiation into cardiomyocytes. Functionally, Bvht appears to act in trans, by interacting with SUZ12, an important component of the PRC2 complex. Interestingly, many of the key TFs within the cardiac gene regulatory network are targets of PRC2 [56]. Cardiogenic differentiation therefore requires the selective loss of PRC2 binding at subsets of these core regulatory TFs, including Nkx2-5, Gata6, Hand1, Hand2, and importantly Mesp1. Since Bhvt depletion maintains enrichment of PRC2 and its associated histone modification, H3K27me3, at the promoters of these critical cardiac TFs, it suggests that Bvht may function as a decoy for PRC2 to promote activation of cardiac lineage-determining genes. These initial findings support the notion for Bhvt, and lncRNAs in general, to be powerful regulatory molecules capable of inducing cardiac specification and differentiation. However, caution should be taken as a conserved Bhvt transcript has not been identified in humans, suggesting that other convergent mechanisms exist.

Another regulator of embryonic heart development has also recently been described. Fendrr (Foxf1 adjacent noncoding developmental regulatory RNA; ENSMUSG00000097336) is specifically expressed in the lateral plate mesoderm of the

developing embryo [57]. Fendrr-expressing cells ultimately give rise to structures of the ventral body wall and the heart. Integration of a premature PolyA signal to disrupt the Fendrr transcript in transgenic mice resulted in embryonic lethality as a consequence of ventral body wall defects and hypoplastic cardiac ventricles. As observed with Bvht, cardiac lineage-determining TFs, including Gata6 and Nkx2-5, were differentially modulated in Fendrr-null hearts. Consistent with a role as an enhancer-associated lncRNA with cis-regulatory action, the proximal TF Foxf1a was ectopically expressed in Fendrr-null embryos. Furthermore, Fendrr was shown to interact with both activating (Trithorax) and repressive (PRC2) chromatin modifiers, targeting these complexes to the promoters of key TFs controlling mesodermal and cardiac specification. Therefore, the capacity to guide cardiac specification makes lncRNAs such as Bvht and Fendrr interesting targets for inducing regeneration. For instance, direct reprogramming of non-myocyte cells into cardiomyocytes, a promising therapy currently under investigation, is associated with differential methylation of H3K27 at cardiac coding gene promoters, a process under the control of PRC2, potentially implicating elncRNAs in cardiomyocyte reprogramming.

Our laboratory has recently assessed transcription from bona fide developmental cardiac enhancers that have been identified using an epigenomic screen in developing embryos [38,49]. The enhancer nature of the identified sequences was previously confirmed in transgenic LacZ reporter mice. Individual sequences exhibit enhancer activity specifically within the embryonic heart and are dynamically transcribed during cardiac differentiation. In addition, elncRNA expression correlates expression of neighboring genes, consistent with cis-regulation. Importantly, depletion of elncRNAs results in reduced expression of enhancer target genes. For instance, activity at the enhancer named mm85 produces an associated transcript, which controls expression of the adjacent gene Myocardin. Myocardin is an important co-factor for serum response factor (SRF), a critical TF implicated in cardiac differentiation [58]. Controlled modulation of Myocardin expression could be envisaged through the manipulation of this elncRNA. In a regenerative context, identification of lncRNAs with similar characteristics could provide novel therapeutic avenues to control precursor cell differentiation into particular cardiac cell fates. To identify elncRNAs more systematically, very deep RNA-Seq was executed on Poly(A)+ RNA derived from differentiating mouse ES cells [49]. Ab initio transcript reconstruction identified hundreds of multi-exonic lncRNAs derived from developmental enhancers undergoing specific state transitions during cardiac specification and differentiation. These findings have been confirmed by other studies [59,60]. Altogether, this suggests that the newly identified elncRNAs represent interesting candidates to control stage-specific differentiation of pluripotent stem cells and/or resident cardiac precursor cells into cardiomyocytes.

Enhancer-associated long noncoding RNAs in the stressed heart

Considering the emerging roles of enhancer-associated lncRNAs in numerous pathological settings, the identification and characterization of these transcripts in the diseased heart is of particular interest. Indeed, the default repair pathways in the adult heart lead to the formation of a fibrotic scar and little regeneration. Since these pathways are controlled by gene regulatory networks underpinned by enhancers and associated elncRNAs, characterizing these transcripts could offer a means to shift the repair regulatory network toward a more regenerative avenue. As a first step toward this, we have recently investigated the mouse long noncoding transcriptome after myocardial infarction using high-throughput RNA-Seq followed by de novo computational reconstruction of the mouse transcriptome [32]. Approximately 1500 novel lncRNAs were identified. Publicly available data sets were then integrated to functionally annotate newly identified lncRNAs. The vast majority were derived from active heart-specific enhancers. Those lncRNAs that were significantly modulated post-myocardial infarction were even more enriched at active cardiac-specific enhancers, implicating these transcripts in the global transcriptional reprogramming that underpins pathological remodeling. In addition, using novel computational approaches, many functions were inferred for these lncRNAs based on their demonstrated chromatin and enhancer state transitions during the stepwise differentiation of mouse ES cells into cardiomyocytes. The larger part of the newly discovered lncRNAs was implicated with cardiac developmental, structural, and functional gene programs. Bearing in mind that reactivation of the fetal gene program is a hallmark of the stressed heart; it is likely that lncRNAs that are modulated upon damage activate specific biological processes as an attempt to induce developmental programs. Targeting elncRNAs could therefore promote the dormant regenerative potential in the injured myocardium. Indeed, one such lncRNA Novlnc6 was shown to be associated with key chromatin-state transitions linked to developmental and maturation cardiac gene programs. Furthermore, modulation of this elncRNA directly impacted two critical cardiac gene regulatory proteins, namely the signaling protein BMP10 and the key cardiac TF Nkx2.5. Importantly, hundreds of predicted human orthologs of heart-specific mouse elncRNAs were identified. Their expression was differentially modulated in human cardiac pathologies, such as dilated cardiomyopathy and aortic stenosis, demonstrating that many lncRNA-mediated gene networks were conserved in humans. Indeed, a comparable study has used RNA-Seq for comprehensive cardiac lncRNA profiling in a cohort of patients suffering with ischemic and non-ischemic heart disease [61]. From a regulatory perspective and cogent with previous findings in mouse, data suggested enhancer-like cis-regulatory interactions between the lncRNAs and their nearby genes, rather than trans-regulatory function with distant genes.

Many cardiac enhancers and super-enhancers are not intergenic but also located within genes. Such intragenic enhancers are able to produce functional elncRNAs. An important cardiac structural gene myosin heavy chain 7 (Myh7) has been recently shown to produce a lncRNA named Myheart (Mhrt), implicated in maintaining function in the stressed heart [62]. This lncRNA is extremely abundant in adult mouse and human hearts. Pathological stress inhibits Mhrt expression in the remodeling heart. This repression is critical for cardiomyopathy to develop, and forced expression of Mhrt is sufficient to protect the heart

from hypertrophy and failure. Mechanistically, Mhrt appears to function by antagonizing the activity of Brg1, a chromatin-remodeling factor previously shown to be responsible for the aberrant activation of gene expression programs leading to remodeling and heart failure [63]. Mhrt acts as a molecular decoy, preventing Brg1 from recognizing and binding its genomic DNA targets. Interestingly, human MHRT is significantly depleted in the hearts of cardiac patients, supporting a conserved role in human cardiomyopathy.

Enhancer-associated long noncoding RNAs in cardiac regeneration

Our current understanding of the biology of elncRNAs supports that they represent a new class of molecules playing key roles in many processes relevant for regeneration, including specification, differentiation, and reprogramming. A comprehensive identification and characterization should therefore be executed across different developmental stages and pathological conditions to identify the most relevant candidates. In particular, such approaches should compare different models of cardiac regeneration to identify conserved orthologous regenerative pathways. For instance, it could be interesting to identify orthologous lncRNAs that are differentially modulated in the Zebrafish heart after ventricular resection or in the neonatal mouse heart following damage, two situations in which regeneration relies on proliferation of dedifferentiate or immature cardiomyocytes [10–12]. Comparing elncRNA profiles of orthologs among vertebrates at corresponding developmental time points will identify lncRNAs with conserved regulatory functions that can be translated in human. Then, controlled cardiogenic differentiation of various stem cell types provides model systems in which to discover new lncRNAs. For example, one could identify elncRNAs, whose expression correlates with master regulators of heart development such as Mesp1, Nkx2.5, and Gata4. Finally, direct reprogramming of non-myocyte cells such as cardiac fibroblasts into cardiomyocytes represents an exciting new paradigm for cardiac regeneration [13,29]. Within this context, considering the potential of lncRNAs as reprogramming factors, profiling the enhancer-templated long noncoding transcriptome to identify elncRNAs that contribute to this process should be envisaged. Considering the exquisite cell-, tissue-, and context-specificity of elncRNAs, systemic modulation of gene regulatory networks that harbor them could be efficiently achieved using targeted depletion with modified antisense oligonucleotides (ASOs) [64]. Such therapeutic approaches would minimize detrimental off-target effects due to intrinsic high specificity of lncRNA expression. Furthermore, mechanisms of action of modified ASOs, when compared to retrovirus-based strategies, do not involve genomic insertion or recombination, making these therapeutic agents ideal for translation in the clinic.

Conclusion

We have only recently entered a new era where technology is forging new frontiers for our understanding of the complex world of regulatory lncRNAs. The new regulatory layer of RNAs exhibits integrated activity with other gene regulatory networks to increase performance and robustness. Enhancer-associated lncRNAs contribute to enhancer activity and thereby to targeted gene expression. The ability to modulate enhancer function via elncRNA knockdown therefore provides a means for controlling temporal coding gene expression in vivo in a highly cell-specific manner. In heart failure patients, expression of relevant protein-coding genes could be altered through controlled expression of elncRNAs and improve outcome. For instance, inflammation could be moderated after myocardial infarction by inducing changes in specific lncRNA expression in inflammatory cells. RNA molecules such as Myhrt could be therapeutically targeted to control cardiomyocyte hypertrophy. Similarly, expression of cardiac fibroblast-specific elncRNAs could potentially be modulated to reduce fibrosis. Finally, through manipulating this new class of regulatory molecules, it might also be possible to switch the pathological reparative response to more regenerative healing processes. It is therefore important to understand the language and function of cis- and trans-acting elncRNAs. Once this is achieved, we hope to gain insights into molecular mechanisms controlling tissue repair and identify new targets for therapeutic intervention. The field of elncRNAs in cardiac disease and regeneration has unprecedented potential for discovery and will no doubt remain a rich field of research for the coming years.

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