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Enhancer RNAs: A missing regulatory layer in gene transcription

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Enhancers and super-enhancers exert indispensable roles in maintaining cell identity through spatiotemporally regulating gene transcription. Meanwhile, active enhancers and super-enhancers also produce transcripts termed enhancer RNAs (eRNAs) from their DNA elements. Although enhancers have been identified for more than 30 years, widespread transcription from enhancers are just discovered by genome-wide sequencing and considered as the key to understand longstanding questions in gene transcription. RNA-transcribed enhancers are marked by histone modifications such as H3K4m1/2 and H3K27Ac, and enriched with transcription regulatory factors such as LDTFs, P300, CBP, BRD4 and MED1. Those regulatory factors might constitute a Mega-Trans-like complex to potently activate enhancers. Compared to mRNAs, eRNAs are quite unstable and play roles at local. Functionally, it has been shown that eRNAs promote formation of enhancer-promoter loops. Several studies also demonstrated that eRNAs help the binding of RNA polymerase II (RNAPII) or transition of paused RNAPII by de-association of the negative elongation factor (NELF) complex. Nevertheless, these proposed mechanisms are not universally accepted and still under controversy. Here, we comprehensively summarize the reported findings and make perspectives for future exploration. We also believe that super-enhancer derived RNAs (seRNAs) might be informative to understand the nature of super-enhancers.

enhancer RNAs (eRNAs), enhancers, super-enhancers, super-enhancer RNAs (seRNAs), gene transcription

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Introduction

As one of the outstanding mysteries in modern biology, the dynamic communication between enhancers and their targeted promoters determines the specification of cellular identity (Levine et al., 2014). It has been more than 35 years since that the enhancer was experimentally demonstrated. Scientists around the world then made extensive efforts to understand the functional interaction between two DNA fragments (i.e., enhancer and promoter) (Bales, 1990; Banerji et al., 1981; Henley et al., 1990). Although significant progress has been made in understanding such interaction, it is still largely unknown how enhancers exactly regulate remote promoters very likely due to unidentified entities (Plank and Dean, 2014; Zabidi and Stark, 2016). Therefore, recently identified enhancer RNAs (eRNAs) become one of the most interesting candidates in the regulation of functional interaction between enhancers and promoters (Kim et al., 2010; Koch et al., 2011). Appreciated with several keynote findings, it becomes clear that enhancers not only regulate the transcription of targeted gene(s) but also actively transcript into eRNAs (Kim et al., 2010; Lam et al., 2014; Liu, 2017). eRNAs, as the name indicates, are transcribed from putative enhancer regions characterized by high levels of H3K4me1, H3K4me2 and H3K27Ac (Djebali et al., 2012; Heintzman et al., 2009; Kaikkonen et al., 2013). They

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exhibit a 5′ cap but are generally not spliced or polyadenylated (Djebali et al., 2012; Lam et al., 2014) (Figure 1). The majority of reports have demonstrated the critical role of eRNAs in transcription of targeted genes. However, the molecular mechanisms are controversial (Li et al., 2013; Melo et al., 2013; Rahman et al., 2017).

In a given status, there are thousands of active enhancers, of which only a very small fraction can be subdivided into a distinguished group (called super-enhancers, SEs) based on the intensity of H3K27Ac or BRD4 binding (Chapuy et al., 2013; Whyte et al., 2013). To date, it is still unclear whether SEs represent a new entity or just clusters of conventional enhancers (Cheng et al., 2016; Dukler et al., 2017; Li et al., 2018; Pott and Lieb, 2015). Like enhancers, super-enhancers also transcribe into RNA which is named as super-enhancer RNAs (seRNAs). It is unclear whether seRNAs represent a new entity or is just a subtype of conventional eRNAs (Alvarez-Dominguez et al., 2017; Micheletti et al., 2017). In this review, we make a comprehensive summary of the reported literature, and provide several perspectives for future studies as well as the potential unique features of super-enhancers and seRNAs. The implications for correlative diseases are also discussed.

The discovery, definition and characteristics of eRNAs

In 2010, two independent studies using RNA-sequencing demonstrated the existence of widespread transcription at active enhancers (De Santa et al., 2010; Kim et al., 2010). Although the technique of RNA-sequencing was widely used, eRNA was missed due to low sequencing depth and focusing on polyadenylated RNAs. In the following few years, a number of groups not only confirmed the existence of eRNAs, but also showed the critical role of eRNAs in gene transcription (Koch et al., 2011; Kowalczyk et al., 2012; Wang et al., 2011). The production of eRNAs is a bona fide reflection of enhancer activation and its signature can be used to predict tissue-specific enhancers independent of any known epigenomic enhancer markers (Cheng et al., 2015; Wu et al., 2014; Yao et al., 2015). When looking back, actually there are several indicators showing the existence of eRNAs decades ago. For example, RNA labeling showed that a large fraction of nascent RNA retains in the nucleus and this nuclear RNA is very unstable compared to cytoplasmic RNA (Harris, 1959). Furthermore, several extensively studied enhancers such as LCR of beta-globin region and enhancers between *DLX5/6* genes show the existence of transcripts(Collis et al., 1990; Feng et al., 2006), but these few examples at that time were insufficient to distinguish eRNAs as a new subset from long non-coding RNA. The ENCODE consortium (2012) has identified 400,000 putative enhancers in human genome, and this number will very likely increase to as many as a million, which is largely extended to the number of 25,000 genes encoding by same human genome. Due to the widespread transcription of enhancers, the number of eRNAs will be very impressive accordingly. Unlike promoters of long non-coding RNAs (lncRNAs) and genes, enhancers show little biasness in the direction of transcription initiation and is frequently bi-directional transcribed (Djebali et al., 2012; Koch et al., 2011; Lam et al., 2014; Natoli and Andrau, 2012) (Figure 1). After transcription, generally, eRNAs do not undergo full maturation processes, they are with 5′ cap but without splicing and polyadenylation (Djebali et al., 2012; Koch et al., 2011; Lam et al., 2014; Natoli and Andrau, 2012). In addition, eRNA is retained in nuclei and is very unstable and can be degraded within minutes (Figure 1). These features indicate that it might only play roles from a limited distance.

The transcriptional regulation of eRNAs

The eRNAs are widely transcribed from enhancers and these enhancers have following features: (i) characterized by high level of H3K4me1, H3K4me2, H3K27Ac but with low level of H3K4me3 and H3K27me3; (ii) bound by lineage-determining transcription factors (LDTFs); (iii) associated with transcriptional co-regulators including subunits of Mediator, histone acetyltransferase P300 and cAMP response element-binding protein (CREB) binding protein (CBP); (iv) occupied by transcriptional initiation complex and phosphorylated RNAPII at serine 5 but not serine 2; (v) binding with condensing complex including enhancer-associated coactivators/corepressors to permit eRNA transcription (Li et al., 2015); (vi) upon stimulation, enhancers are occupied by DNA topoisomerase I (TOP1) within minutes and the DNA nicking activity of TOP1 is a prerequisite for robust eRNA synthesis (Puc et al., 2015) (Figure 1). Collectively, the enriched transcriptional regulatory factors at enhancer might constitute a Mega-Trans-like complex to potently activate enhancers (Liu et al., 2014) (Figure 1). Furthermore, the enhancer DNA that produces eRNA exhibits low DNA methylation and enrichment of DNA hydroxylase Tet1 (Pulakanti et al., 2013).

It was believed that H3K4me1and H3K27ac are markers of active enhancers. However, it is surprising to see minor effects on eRNA transcription after losing H3K4me1 and partially reducing H3K27ac in CRISPR-CAS9 engined Mll3/4 catalytically deficient cells (Dorighi et al., 2017). In contrast, loss of Mll3/4 proteins leads to strong depletion of enhancer RNAPII occupancy and eRNA synthesis. Thus, Mll3/4 protein but not their enzymatic activity is critical for eRNA synthesis at enhancer. The observed enrichment of

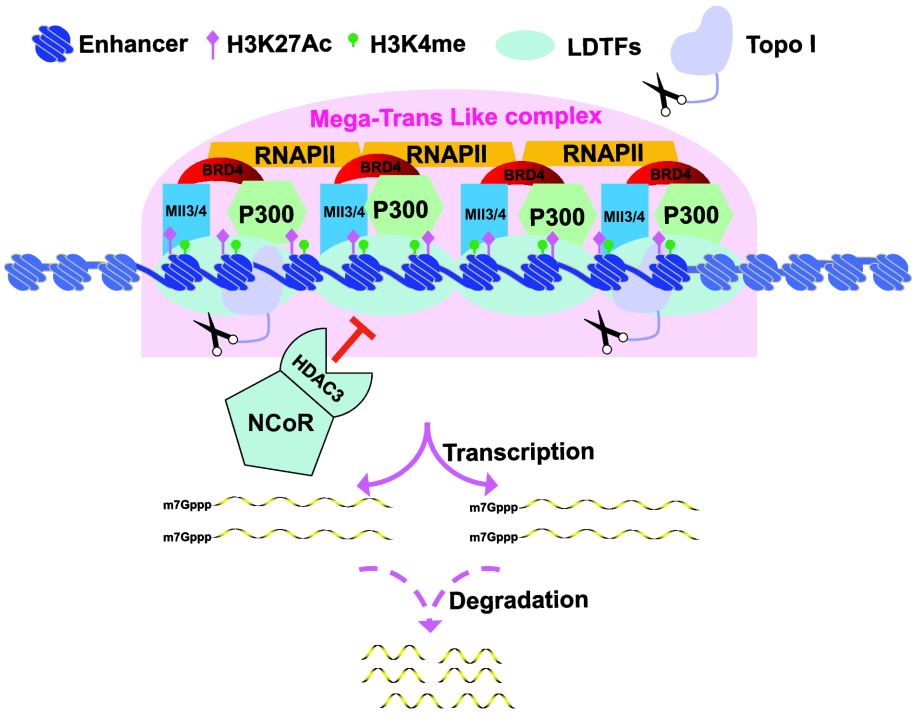

Figure 1 Transcriptional regulation of eRNA production. The eRNA producing enhancers show high level of histone modification of H3K4me1, H3K4me2, H3K27Ac and are enriched with transcription regulators such as LDTFs, P300, CBP, BRD4, MED1 and TOP1. These enhancer-binding factors collectively comprise a Mega-Trans like complex, which can potently induce the expression of eRNAs. Induced eRNA is unstable due to lack of polyadenylated tails and they might only play roles at local.

H3K4me1 at active enhancer might be noise and their bone fide role in eRNA production needs further investigation.

Besides these commonly regulated mechanisms, several proteins favor to bind at enhancer regions and subsequently regulate the transcription of enhancers. A recent study showed that a part of p53 binds at enhancer regions, which is required for eRNA production at such regions (Allen et al., 2014; Léveillé et al., 2015; Melo et al., 2013). Actually, p53 actives LED (lncRNA activator of Enhancer Domains) to turn on enhancers, which could amplify a subset of p53-dependent eRNA production. It also has been shown that stimulation triggered integrator binds to enhancers and is required for eRNA production(Allen et al., 2014; Léveillé et al., 2015; Melo et al., 2013). Upon pro-inflammatory stimulation, TEAD/P65 complex could induce eRNA at MnSOD locus (Chokas et al., 2014). What's more, transcription factor FOXO3 selectively binds to enhancer regions and promotes enhancer transcription. Although these above factors have shown to enhance eRNA production, their molecular mechanisms are largely unclear. Comparing to positive regulators, the negative regulators are even less determined. The 7SK-BAF axis has shown to inhibit eRNA production (Flynn et al., 2016). Several nuclear receptors such as Rev-Erb-a and Rev-Erb-b could recruit nuclear receptor co-repressor (NCoR)-HDAC3 complexes to turn off eRNA production (Lam et al., 2014) (Figure 1). In addition, compared to transcription regulation of promoter upon signaling induction, the transcriptional regulation of eRNA upon stimulation is largely unknown. The understanding of eRNA transcription at molecular level will help us to understand how does enhancers' function precisely regulated.

The functions and molecular mechanisms of eRNAs

Generally, the expression level of eRNAs is correlated with expression of their nearby genes, which suggests their potential important role in gene transcription. The first evidence to prove the important function of eRNA was from the studies of p53. P53 binds to both promoter and enhancer regions. P53 binding enhancers actively transcribed eRNA which are required for stress-induced p53 function (Allen et al., 2014; Leveille et al., 2015; Melo et al., 2013). Using estrogen (or androgen)/ER system, the detailed molecular mechanism of eRNA was probed (Li et al., 2013). Estrogen-induced eRNA exerts important roles in estrogen-upregulated coding genes via cohesion-dependent mechanism to increase the strength of specific enhancer-promoter looping (Hah et al., 2013; Li et al., 2015) (Figure 2A). The androgen induced eRNAs such as KLK3e, transcribed from upstream enhancers of AR-regulate gene KLK3, not only facilitate the spatial interaction between KLK3 enhancer and KLK3 promoter but also increase long-distance KLK2 transcriptional activation (Hsieh et al., 2014). Depending on the integrity of

core enhancer elements, KLK3e processes RNA-dependent enhancer activity and is required for the interaction of AR and Mediator 1 (Med1) (Hsieh et al., 2014). Androgen induced prostate-specific antigen (PSA) eRNA binds to CYCLIN T1, activates P-TEFb and promotes *cis* and *trans* target gene transcription by increasing serine-2 phosphorylation of RNA polymerase II (Zhao et al., 2016). The CDK9 inhibitor flavopiridol blocks eRNA synthesis but does not affect other markers of enhancer activity, suggesting eRNA systhesis after assembly of active enhancers (Hah et al., 2013) (Figure 2).

In a similar system at single cell level, eRNAs rarely co-express with their target loci (Rahman et al., 2017), which indicates that active gene transcription does not require the continuous transcription of eRNA or their accumulation at enhancers. It suggests, eRNAs engage at very early phase of gene transcription after enhancer assembly, which is consistent with a genomic study indicated that eRNA transcription is the earliest response, even prior to transcription of messenger RNAs encoding transcription factors (Arner et al., 2015)

Although some evidences suggest that eRNAs affect enhancer-promoter looping, their putative role in gene transcription is still under debate. One study shows that eRNAs do not affect chromatin looping, but act as a decoy for the negative elongation factor (NELF) complex and thus facilitate the transition of paused RNA polymerase II into productive elongation (Schaukowitch et al., 2014) (Figure 2C). Another study demonstrates that eRNAs increase pol II binding (Maruyama et al., 2014) (Figure 2C). Besides these restricted effect, several other studies have showed that eRNAs owe broad effect on chromatin states (Liang et al., 2016; Pnueli et al., 2015; Yang et al., 2016). Knockdown eRNA results in an increase in total histone H3 occupancy at enhancer region and a virtual loss of H3K4m3 at promoter region (Liang et al., 2016 ; Pnueli et al., 2015; Yang et al., 2016) (Figure 2B). eRNA could also open chromatin directly or through recruitment of chromatin remodelers (Mousavi et al., 2013) (Figure 2B). A recent study demonstrates that the eRNA, ThymoD, could reposition its targeted Bcl11b enhancer from the lamina to the nuclear interior (Isoda et al., 2017). However, models proposed above are not universally accepted, by using different stimulation system, the mechanism by which eRNA regulates targeted gene expression might be quite different. The underlying nature of eRNA in the regulation of gene expression is still unclear, which needs further investigation.

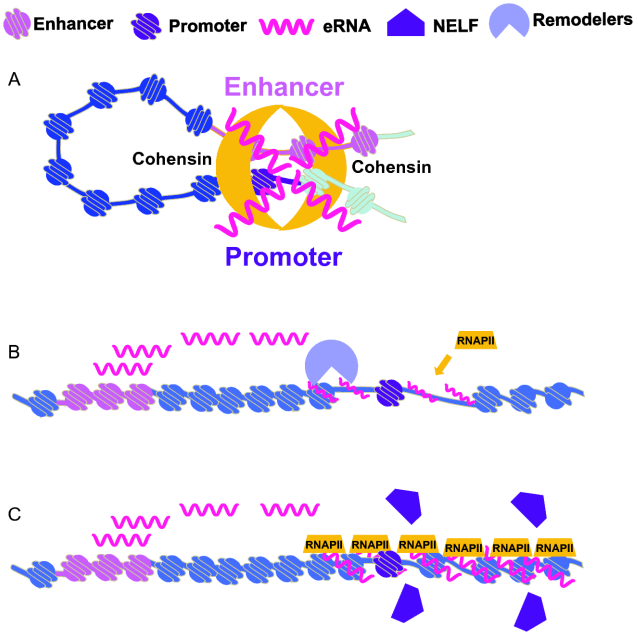

Figure 2 Models to show how eRNAs regulate the transcription of targeted genes. A, After signal induced transcription of eRNAs, they facilitate functional interaction between enhancers and promoters, then further hold and stable the chromatin loop via cohensin dependent or independent pathways. B, Enhancer RNAs initiate the transcription of targets by opening chromatin directly or indirectly via recruitment of chromatin remodelers. C, Enhancer RNAs promote targets transcription by enhancing the binding of RNAPII or leasing negative regulator NELF.

eRNA in diseases

Following the discovery of eRNA, extensive efforts have been made to understand the function of eRNAs and their molecular mechanism in regulation of gene transcription. However, their potential pathologic roles in diseases remain obscure. Genome wide association studies revealed that majority of diseases associated with single nucleotide polymorphisms (SNPs) locate outside of protein coding regions of the human genome (Maurano et al., 2012). Epigenomic profiling further showed that many of non-coding risk SNPs fall within the tissue specific enhancers (Akhtar-Zaidi et al., 2012; Andersson et al., 2014; Corradin et al., 2014; Ernst et al., 2011). Besides SNPs, somatic mutations in noncoding intergenic elements introduce new super-enhancers to drive the expression of oncogenes in cancer (Mansour et al., 2014). Likewise, small genomic insertions in noncoding regions also form new enhancers or super-enhancers to upregulate expression of key oncogenes in different cancers (Abraham et al., 2017). Due to the fact that eRNAs are widely transcribed at enhancers, a part of enhancer-risk SNPs could change the sequence of eRNAs and subsequently affect its function, which then cause diseases. The altered genomic regions through somatic mutations or insertions also very likely affect their transcripts. In patients with non-medullary thyroid carcinoma (NMTC), an eRNA is greatly down-regulated because of a single-nucleotide mutation in the enhancer region (He et al., 2013). In mouse models of Huntington's disease, the expression of eRNAs also largely reduces (Le Gras et al., 2017). Enhancer RNAs might also

contribute to hematopoiesis, carcinogenesis and drug-resistance (Jeong and Goodell, 2016; Smith and Shilatifard, 2014; Zhao et al., 2016). As the prominent function of enhancers in both physiological and pathological conditions, we hypothesize that eRNAs contribute largely in a wide spectrum of diseases which deserve further investigation. The knowledge of eRNAs not only provide new insight to understand gene transcription but also light up a new way in understanding diseases. The challenge is how we can distinguish the causal roles between enhancer and eRNA. Some of genetic alternation might affect both enhancer and eRNA function, but some of them might only affect eRNA but not enhancer function.

eRNA versus super-enhancer RNA

Enhancers are regular segments of DNA that are a few hundred base pairs in length generally and can be bound by proteins to increase the likelihood of transcription of particular genes (Blackwood and Kadonaga, 1998; Pennacchio et al., 2013) (Figure 3). Similar to enhancer, the length of eRNA, transcribed from enhancer, is also generally a few hundred base pairs. A recent distinguished subset from enhancer is termed super-enhancer based on the intensity of transcriptional factor binding and histone modification (Whyte et al., 2013) (Figure 3). Compared to enhancers, they are more powerful to regulate gene expression (Whyte et al., 2013) and larger in size with general size of about 8 kb (Whyte et al., 2013). Besides these known differences between enhancer and super-enhancers, it is continuing controversy over whether super-enhancers genuinely represent a new paradigm in transcriptional regulation or just be a cluster of conventional enhancers (Dukler et al., 2017; Pott and Lieb, 2015). Such critical question might be addressed by considering RNA transcription from super-enhancers (seRNAs) (Figure 3). Unlike eRNAs, seRNAs are even larger due to the large size of super-enhancers. Furthermore, when analyzing the fraction of eRNAs that overlaps with TEs or SEs, relatively few typical intergenic enhancers overlap with eRNA (30.6%), whereas nearly all intergenic SEs contain eRNAs (93.3%) (Hah et al., 2015) (Figure 3). It suggests that active transcription is another feature of super-enhancers. More importantly, majority of seRNAs are capped and polyadenylated RNAs (Alvarez-Dominguez et al., 2017), indicating that seRNAs are more stable and have more broad and profound effect in physiological and pathological conditions. Thus, seRNAs are not simply the sum of eRNA transcribed from corresponding individual enhancers (Figure 3). It is different from eRNA at several aspects such as expression level, length, modification, or even nucleotide sequence (Figure 3). More studies of seRNAs will be able to understand the nature of seRNAs and SEs. Beyond the cis-acting effect of typical enhancer, super-enhancer might also have trans-acting effect through transcription of seRNAs (Alvarez-Dominguez et al., 2017).

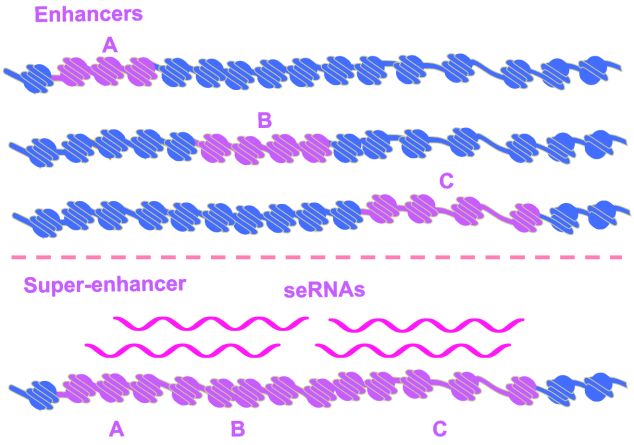

Figure 3 A model by using seRNAs to show super-enhancers genuinely represent a new paradigm in gene transcription but not just be clusters of conventional enhancers. Although a super-enhancer is composed of a cluster of enhancers, it is a new functional entity instead of the sum of individual constitutive enhancers. These super-enhancers comprised enhancers are small in size and they are active individually in certain situation. But these individually active enhancers do not produce eRNAs. When all enhancers are active, they constitute the super-enhancer and become a new unit, and subsequently produce lots of large seRNAs to regulate different targeted genes.

Perspectives

Recently, the enhancer RNAs have become one of the most interesting fields in the understanding of gene transcription due to their wide transcription in universal conditions. Large scale genome-wide sequencing has identified thousands of eRNAs in different conditions. The wide expression in each condition suggests potential important role of eRNAs in gene transcription. Although few studies report that there is no significant role of eRNA in nearby gene expression, majority of functional studies have demonstrated critical role of eRNAs in nearby gene expression. Nevertheless, when considering the 3D nuclear structure and DNA architectures in nucleus, eRNAs may not only play a role in linear nearby gene expression, but also affect the linear distant but spatial nearby gene expression. This possibility may explain why knockdown eRNA expression in few studies has minimal effect in linear nearby genes. Genome-wide analysis of gene expression after depletion of individual eRNAs will help to understand eRNA-regulated genes in genome wide scale and also provide useful information in understanding 3D nuclear structure since eRNAs are unstable and affect gene expression locally. One of prominent question is how eRNAs regulate gene expression: whether eRNAs affect enhancer-promoter looping? Whether eRNAs recruit transcription

regulators to target genes? How eRNAs exert its function with perfect timing? Whether eRNAs limit targeted gene expression? Furthermore, as reported eRNAs also regulate gene splicing through recruiting splicing complex, interaction between eRNAs and enhancers might also introduce DNA instability. It is therefore important for understanding carcinogenesis, aging and other diseases that associated with DNA instability. At last, with our full understanding the biological function of eRNAs, clinical application using eRNA as a biomarker might be better than frequently used biomarkers such as mRNA, DNA or protein. In summary, we are just beginning to touch the newly identified entity, eRNA, which is in transient existence, but might affect not only gene transcription but also RNA metabolism as well as nuclear architecture.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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