REVIEW ARTICLE

Novel Approaches to Profile Functional Long Noncoding RNAs Associated with Stem Cell Pluripotency

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Abstract: The pluripotent state of stem cells depends on the complicated network orchestrated by thousands of factors and genes. Long noncoding RNAs (lncRNAs) are a class of RNA longer than 200 nt without a protein-coding function. Single-cell sequencing studies have identified hundreds of lncRNAs with dynamic changes in somatic cell reprogramming. Accumulating evidence suggests that they participate in the initiation of reprogramming, maintenance of pluripotency, and developmental processes by cis and/or trans mechanisms. In particular, they may interact with proteins, RNAs, and chromatin modifier complexes to form an intricate pluripotency-associated network. In this review, we focus on recent progress in approaches to profiling functional lncRNAs in somatic cell reprogramming and cell differentiation.

Keywords: Pluripotency, stem cells, long noncoding RNA, reprogramming, epigenetics, RNA sequencing.

1. INTRODUCTION

The majority of eukaryotic genomes consist of noncoding RNA (ncRNA), having no protein-coding function. The ENCODE project suggests that non-coding transcripts account for 62%-75% of our genome [1, 2]. They were originally regarded as transcriptional junk. However, accumulating evidence has demonstrated that these noncoding RNAs are important biomarkers with diagnostic value for the prognosis of inflammation-related alterations [3]. Long noncoding RNAs (lncRNAs) of more than 200 nt in length are one of the two major ncRNAs. Owing to the development of next-generation sequencing techniques, especially RNA sequencing (RNA-seq), new functional lncRNAs have been discovered and annotated each year [4]. According to the NONCODE database with the most comprehensive number of transcripts to date, there are 96308 and 87774 lncRNA genes in humans and mice, respectively. Most annotated lncRNAs are transcribed by Pol II. Hence, they are presumably capped, polyadenylated, and spliced similarly to mRNAs [5-7]. These lncRNAs exhibit more tissue-specific expression than protein-coding genes and have potential as gene regulators participating in inflammation-related diseases [8-10].

Pluripotent stem cells have potential applications in developmental biology and regenerative medicine [11, 12]. There are two main types of pluripotent cells: embryonic stem cells (ESCs) that are directly isolated from blastocysts, and induced pluripotent stem cells (iPSCs) that are induced by reprogramming transcription factors. To effectively use these cells for clinical therapy, researchers have invested great effort in their study and made great progress in the past decades.

Accumulating evidence indicates that lncRNAs are important regulators of stem cell pluripotency [13-15]. Several studies have characterized the transcriptome of ESCs, suggesting that lncRNAs regulate the pluripotent state [14, 16, 17]. Researchers have identified certain lncRNAs that play critical roles in maintaining pluripotency or promoting cell lineage differentiation. To better manipulate cell fate and improve reprogramming efficiency, identification of lncRNAs as well as their functions and mechanisms is crucial. Currently, predicting which lncRNAs are functional and their potential functions is difficult. Thus, a systematic approach to evaluate the function of cell type-specific lncRNAs is important to understand the roles of lncRNAs in pluripotency. In this review, we first focus on the current approaches and methods to annotate lncRNAs related to pluripotency control.

2. MAPPING PLURIPOTENCY-ASSOCIATED lncRNAs

2.1. Microarray and RNA-seq

With the mounting evidence of lncRNAs playing important roles in the regulation of cell fate, researchers have begun to study lncRNAs in ESCs and iPSCs. First, they used

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microarray and RNA-seq technologies to detect lncRNAs that were differentially expressed in ESCs and fibroblasts. Dinger *et al.* used their custom-designed microarray to profile lncRNAs expressed differentially during embryoid body differentiation and identified 174 lncRNAs that may correlate with pluripotency [13]. Because of the characteristics of lncRNAs, their transcriptomes can be reconstructed by RNA-seq using the poly(A) tail or rRNA-depleted total RNA [18]. Single-cell RNA-seq revealed lncRNAs as expressed specifically in various cell lines, including pluripotent stem cell lines [19-22]. Previous RNA-seq studies were mainly dependent on existing annotations and focused on the expression level and transcript variants in known sequences [19, 21, 23, 24]. Guttman *et al.* used published data from ESCs and identified 118 lincRNAs that bound to Oct4 and Nanog promoters based on a chromatin state map [25]. Furthermore, they provided a new method to reconstruct transcriptomes from RNA-seq reads and the genome sequence, and identified an additional 591 lincRNAs in ESCs [22]. Compared with the traditional RNA-seq assay, this new method identifies transcripts of variable sizes, especially for unannotated genome sequences and millions of relatively short sequence reads. It also detects expression levels and protein-coding capacity [22, 25]. Yan *et al.* applied single-cell RNA-seq to 124 individual cells from human preimplantation embryos and ESCs at different stages and discovered 2733 novel lncRNAs in the sequence data [26]. Some studies have performed single-cell RNA-seq to detect lncRNAs related to pluripotency during defined stages of the reprogramming process [27, 28]. They identified 312 lncRNAs activated during somatic cell reprogramming, of which the functions are largely unknown. High-depth poly(A)$^+$ RNA sequencing performed by Jan *et al.* generated a profile of lncRNAs expressed in mouse ESCs, which were highly associated with pluripotency. Compared with the GENECODE M3 annotation, they identified 772 and 705 lncRNAs expressed in embryonic stem cells and neural progenitor cells, respectively, and 508 of the lncRNAs had different expression levels upon differentiation of ESCs to NPCs [28]. Both of these studies have provided the primary landscape of lncRNAs in stem cells.

2.2. Loss-of-function Assays

Based on lncRNAs that are highly expressed in pluripotent stem cells, to identify functional lncRNAs, researchers have performed loss-of-function studies to detect lncRNAs related to the pluripotency of stem cells. Inhibiting the expression of lncRNA genes directly indicates their influence on stem cell pluripotency in terms of both morphology and gene transcription. Knockdown of such lncRNAs induces loss of the pluripotent state and activation of lineage-specific markers [29, 30]. The depletion of specific gene expression visually reveals the function of lncRNAs, but it is difficult to evaluate [31-33]. Common knockdown methods include RNA interference [14, 16] and the Crispr-Cas9 system [33-36]. Guttman *et al.* used shRNAs to knockdown the expression of 147 lincRNAs and found that 137 of the 147 lincRNAs had a significant influence on gene expression in ESCs. The effects were equal to the well-known regulators of ESCs, and 26 of the lincRNAs facilitated maintenance of the pluripotent state [14]. Kim *et al.* conducted loss-of-

function assays with two to four small interfering RNAs to knockdown lncRNA expression levels in late-stage iPSCs and found that reprogramming-activated lncRNAs regulated lineage-specific gene expression [28]. However, RNA interference may have off-target effects and different knockdown efficiencies [37-39]. A similar method employing programmable nucleases, such as zinc finger and transcription activator-like effector nucleases, can precisely silence target genes in mammalian systems including humans [40, 41]. However, these nucleases are not appropriate for library-scale loss-of-function studies. The Crispr-Cas9 system minimizes off-target effects and precisely changes the lncRNA transcriptional level [42, 43]. CRISPR nuclease (CRISPRn) was developed to identify essential genes that control the viabilities of cancer cells, embryonic stem cells, and human leukemic cells [44, 45]. However, CRISPRn may not be the most useful tool for loss-of-function assays because of low knockout efficiency [46]. Several studies have developed CRISPRi technology and confirmed that this CRISPR system can be used to repress specific gene transcription levels [43, 47, 48]. Using this method, Liu *et al.* found that nine lncRNA loci downregulated pou5f1/Oct4 expression and most of the lncRNAs were involved in primary control of iPSC growth [33]. The depletion of large scale lncRNA expression also remains challenging [49]. One approach to resolve this issue is to remove the promoter region that is shorter and easier to remove compared with entire lncRNA genes. To further study the specific roles of lncRNAs in complex networks, faster and more effective methods to perform loss-of-function assays have been developed recently, which are useful to identify additional pluripotency-associated lncRNAs.

2.3. ChIP Assays

Chromatin is an important controller of reprogramming, reprogramming factor levels, stoichiometry, and extracellular conditions influencing cell fate [50]. The chromatin immunoprecipitation (ChIP) assay was developed to identify interactions between DNA and nuclear proteins including histones and many transcription and post-transcription factors of histones [51]. It can be used directly to probe interactions with candidate regions (ChIP-PCR) or combined with high throughput sequencing (ChIP-seq) to generate genome-wide information. Using this approach, entire locus candidates of specific functional proteins can be mapped. Previously, Guttman *et al.* analyzed ChIP-seq data of trimethylated lysines 4 and 36 of histone H3 in four cell types and identified approximately 1600 large intergenic ncRNAs (lincRNAs) [25]. By comparing with published data from mouse ESCs, they identified 118 lincRNAs for which the promoter loci were bound by core transcription factors OCT4 and NANOG [25, 52]. These studies demonstrate that the ChIP assay is an effective method to identify functional lncRNAs.

2.4. RIP Assay

RNA immunoprecipitation is a technique to detect RNAs related to specific proteins using the corresponding antibody. Some proteins play essential roles in regulating stem cell self-renewal and pluripotency [53-65]. OCT4, SOX2, and NANOG are the three factors that mainly regulate the maintenance of pluripotency, which constitutes the core plu-

ripotency network. In addition, a growing number of proteins have been identified, which positively or negatively modulate the function of the core complex. These functional proteins may regulate the expression of pluripotency genes by epigenetically changing the chromatin structure [66]. In fact, several lncRNAs have been shown to bind to OCT4 [16], SOX2 [17], NANOG [67, 68], and WDR5 [69]. Researchers have begun to identify regulators that bind to functional proteins to perform their epigenetic regulatory function in *trans*. RIP-seq was developed by combining native RIP [56] and RNA-seq [19]. This method further demonstrated that lncRNAs interact with chromatin complexes and are involved in regulating epigenetic markers [14]. Zhao *et al.* first used this method to capture the genome-wide pool bound to PRC2. They also compared the group of intergenic ncRNAs detected by Guttman *et al.* with their RIP-seq data and found 216 overlapping lincRNAs, indicating that these lincRNAs are highly associated with stem cell pluripotency and probably perform their functions by recruiting PRC2 [70]. Many epigenetic regulators, such as Polycomb group proteins and DNA methyltransferases, are critical for stem cell pluripotency and differentiation [65, 71]. Sequencing data and RT-qPCR of RIP can broadly identify functional lncRNAs related to epigenetic regulators of pluripotent stem cells.

2.5. ChRIP Assay

lncRNAs play functional roles in chromatin organization [72-74]. The functionally distinct epigenetic state of chromatin decides the transcription level of pluripotency-related genes. Hence, identification of chromatin-associated lncRNAs in functional chromatin compartments may provide a better profile of pluripotency-associated lncRNAs. Chu *et al.* developed chromatin RNA immunoprecipitation technology to profile chromatin-associated lncRNAs [74]. Modified protocols of ChRIP have emerged and successfully identified a set of lncRNAs [75-79]. ChRIP technology can be used not only to investigate lncRNAs associated with chromatin architecture regulation but also their genome-binding sites and regulatory mechanisms [74, 75, 80, 81]. Two other alternative methods have been published, namely Chart and RAP [78, 80]. These protocols are very similar to ChIRP with some differences in cross-linking, chromatin shearing, and oligonucleotide probe design [81]. The coprecipitated RNA is analyzed by RT-qPCR or high throughput sequencing. Somatic cell reprogramming is always accompanied by dynamic chromatin changes by comprehensive epigenetic modification of regulatory DNA elements. These regulatory DNA elements include histone modifications, promoters, enhancers, transcribed exons, and silent genes [64, 82]. To detect regulatory DNA elements related to lncRNAs, ChIRP-seq is generally applicable to illustrate the intersection of lncRNA and chromatin with newfound precision genome-wide.

2.6. CRIST-seq

The chromatin architecture status determines the transcription level of genes. LncRNAs play their crucial roles as regulators by participating in the orchestration of the three-dimensional chromatin architecture [83, 84]. These chromatin architectures usually consist of chromatin looping structures, lncRNAs, and protein regulators that control the tran-

scriptional state [85]. In pluripotent stem cells, lncRNA-mediated chromatin structures promote the expression of stemness-associated genes by bringing distant enhancer elements close to the core promoter, thereby maintaining the pluripotent state [86]. In recent years, many pluripotency-associated factors, such as POU5f1, SOX2, and NANOG, have been found to be useful to maintain stem cell pluripotency [87-91].

Based on such regulatory mechanisms, novel chromatin *in situ* reverse transcription sequencing (CRIST-seq) approach was developed to detect RNA-related interaction networks [92]. The specific process is shown in Fig. (**1A**). This assay takes advantage of both the specificity of the Cas9 gene-editing system and the simplicity of lncRNA *in-situ* biotin labeling. Target cells are transfected with catalytically inactive CRISPR Cas9 (dCas9)-gRNAs to target a specific site in a gene locus, such as the gene promoter or enhancer. After fixation and sonication, chromatin RNAs are labeled by *in situ* reverse transcription using biotin-dCTP. The Cas9 chromatin complex is immunoprecipitated by an anti-Cas9-FLAG antibody, and the biotin-labeled cDNAs are purified by streptavidin beads for library sequencing. The gene-associated lncRNA network can be mapped specifically over the control. Using this approach, Zhang *et al.* mapped the promoter-interacting lncRNA network for Pou5f1 and Sox2 that are activated during the reprogramming process [92]. By integrating both CREST-seq and RAT-seq datasets, they identified several pluripotency-associated lncRNA candidates including *Platr10* [93], *Spilr9* (*Sox2* promoter-interacting lncRNA 9), and ENSMUSG00000100826 (*Snhg14*, *Spilr14*; *Sox2* promoter-interacting lncRNA 14). Their functions in pluripotency regulation were validated by knockdown and overexpression assays. Delineation of this pluripotency-specific lncRNA-DNA interaction network may facilitate understanding of how a gene and its associated lncRNAs act in concert to control cell fate during reprogramming. Most importantly, this CRIST-seq approach can be broadly used to screen lncRNAs that interact with chromatin regulatory regions such as promoters and enhancers.

2.7. Reverse Transcription-associated Trap (RAT) Assay

There is a comprehensive network maintaining stem cell pluripotency. LncRNAs are usually involved in the complicated regulatory network by building or removing blocks in regulating cell pluripotency or the reprogramming process [16, 94]. They modulate gene expression by various mechanisms such as forming an RNA-DNA triplex that facilitates the recruitment of effector proteins to the gene promoter [95-97]. To better elucidate the relational network of specific lncRNAs with other DNAs, Sun *et al.* first established a novel method to detect target genes of a specific RNA. This method is called RNA reverse transcription-associated trap sequencing (RAT-seq); (Fig. **1B**) [27, 98-100]. At various stages of reprogramming cells, RNA-seq data identifies thousands of differentially expressed lncRNAs. It is assumed that these lncRNAs play crucial roles in establishing the specialized transcriptional network of stem cell pluripotency.

RAT-seq identifies lncRNA-specific regulatory element networks to determine whether a specific lncRNA is associated with core factor genes of stem cells or the pathway
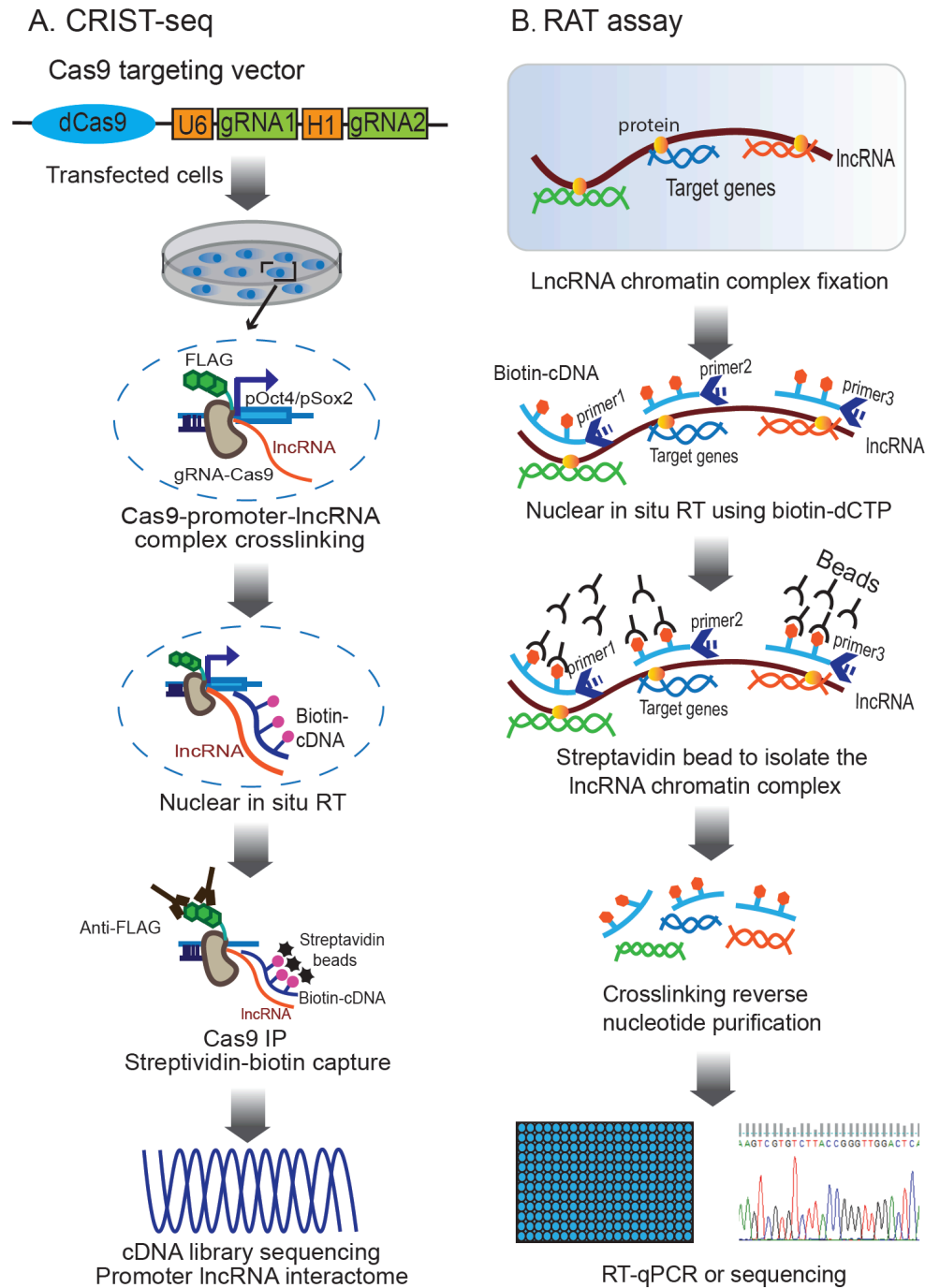
Fig. (1). Schematic diagrams of CRIST-seq and RAT-seq assays. A. Schematic diagram of the CRIST-seq assay. dCas9: catalytically inactive CRISPR/Cas9; gRNA: Cas9-guiding RNAs that target the gene promoter; pU6: RNA polymerase III *U6* promoter; pH1: human *H1* RNA polymerase III promoter. Cells are transfected with the Cas9 gRNA cassette that targets the promoter of a specific gene. In this schematic diagram, *Oct4* and *Sox2* (well-established core stem cell factors required for the maintenance of pluripotency) promoters are used as targets. The Cas9 gRNA-expressing cells are crosslinked by formaldehyde to fix the RNA-DNA structure. After cell lysis, nuclei are isolated and the promoter-interacting RNAs are *in situ* reverse transcribed into cDNAs with biotin-dCTP. The promoter biotin-cDNA chromatin complex is immunoprecipitated by an antibody against FLAG, which binds to its target genes through base pairing between the gRNA and target DNA. After Cas9-FLAG immunoprecipitation, the promoter-interacting biotin-cDNAs are separated from genomic DNAs by streptavidin beads. The CRIST-captured chromatin cDNAs are collected for library construction and sequenced to identify lncRNAs that interact with the promoter of the target gene. **B.** Schematic diagram of the RNA *in situ* reverse transcription-associated trap (RAT) assay. After fixation with formaldehyde, a specific lncRNA is *in situ* reverse transcribed using lncRNA-specific primers and biotin-dCTP. The lncRNA-biotin cDNA chromatin complexes are pulled down with streptavidin beads and the chromatin complex DNAs to which the lncRNA binds are isolated for library sequencing or qPCR. RAT-seq maps genome-wide gene targets for the lncRNA candidate. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

genes that are crucial for reprogramming. Using this method, functional lncRNAs associated with reprogramming can be identified. Du *et al.* integrated the data of RNA-seq and RAT-seq, and attempted to identify lncRNAs that were not only differentially expressed in the somatic cell reprogramming process, but also associated with pluripotency core factor genes or pathway genes [27]. *Platr10*, the identified pluripotency-associated transcript 10 [93], also identified as *Oct4-Sox2*-coating long noncoding RNA 8 (*Osclr8)* by the *CRIST*-seq method [92], was validated to bind to Oct4 and Sox2 promoters by RAT sequencing data [27]. This new approach identifies pluripotency-associated lncRNAs and indicates the mechanisms by which lncRNAs perform their functions including alterations in the three-dimensional chromatin structure, DNA methylation, histone modifications, and enhancer RNAs.

2.8. DNA-RNA Fluorescent *In situ* Hybridization (FISH)

Fluorescent *in situ* hybridization (FISH) is a visual technique used to detect nucleic acids in cells and tissues [101]. It provides a unique method to study gene networks, chromatin organization, and gene expression at the subcellular level. DNA and RNA FISH were developed for different uses in molecular research and gene diagnostics [102], such as assessing a gene expression level [103], subcellular localization [104], cancer diagnostics [105], and karyotyping [106]. Based on directly or indirectly labeled probes, incubating the sample with appropriate fluorophore-labeled antibodies or specific binding molecules may be accomplished. It has been demonstrated that the location of a gene within the nucleus can regulate the transcription level of a specific gene [107]. To further investigate the relationship of the spatial position and transcriptional activity, DNA-RNA FISH was developed [108-110]. The combined DNA-RNA FISH method may be applied to other circumstances where both RNA and DNA need to be detected [110]. LncRNAs regulate the transcription level by *cis* or *trans* manners [7]. In pluripotent stem cells, lncRNAs play important roles in the regulation of pluripotency and differentiation [13]. Researchers are interested in determining whether lncRNAs regulate stemness gene expression by binding to their transcriptional elements, thereby promoting or inhibiting the expression level of pluripotency genes. DNA-RNA FISH is a direct method to visually show DNA and RNA associations. To date, several lncRNAs have been shown to be associated with pluripotency and differentiation by this method [92, 110].

CONCLUSION AND PROSPECTIVES

Pluripotent stem cells, mainly induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs), have raised great hope for regenerative medicine and the treatment of inflammation-related diseases. Due to the application constraint of embryonic stem cells and the advantages of induced pluripotent stem cells, researchers have begun to focus on generating induced pluripotent stem cells with higher efficiency. There is a significant epigenetic roadblock that needs to be addressed before somatic cells are reprogrammed to pluripotent cells, and there is a comprehensive relationship between regulators that maintain pluripotency. Long noncoding RNAs have been shown to play crucial roles in the regulation of pluripotency by versatile mechanisms [9].

Therefore, effective methods to identify functional lncRNAs in the regulation of pluripotency are crucial. These advanced approaches not only identify lncRNAs associated with pluripotency, but also indicate their mechanisms by which they perform their functions.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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