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The essentiality of non-coding RNAs in cell reprogramming

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ABSTRACT

In mammals, short (mi-) and long non-coding (lnc) RNAs are immensely abundant and they are proving to be more functional than ever before. Particularly in cell reprogramming, non-coding RNAs are essential to establish the pluripotent network and are indispensable to reprogram somatic cells to pluripotency. Through systematic screening and mechanistic studies, diverse functional features of both miRNA and lncRNAs have emerged as either scaffolds, inhibitors, or co-activators, necessary to orchestrate the intricacy of gene regulation. Furthermore, the collective characterizations of both miRNA and lncRNA reveal their interdependency (e.g. sequestering the function of the other) to modulate cell reprogramming. This review broadly explores the regulatory processes of cell reprogramming - with key functional examples in neuronal and cardiac differentiations - in the context of both short and long non-coding RNAs.

1. Introduction

1.1. Regenerative medicine and reprogramming of cell state

Restoring normal functionality of diseased or injured tissues with healthy ones forms the crux of regenerative medicine [1]. During the embryonic development, cells terminally differentiate to more specialized cell fates with reduced cellular plasticity. However, numerous reprogramming methods have been developed to achieve pluripotency by reprogramming somatic cell to the pluripotent state or to another lineage [2]. This plasticity of the pluripotent stem cells to attain different cell fates provides a great potential to treat different diseases. Although the transfer of somatic cell nuclei [3] by oocytes [4] or sperm RNA [5] has shown the potential to reprogram cell states, transcription factor mediated reprogramming to generate induced pluripotent stem cells (iPSCs) [6] or mediate direct lineage conversion (*trans*-differentiation) [7] has gained considerable interest.

Introduction of exogenous transcription factors for somatic cell reprogramming leads to gross perturbations of the transcriptome and epigenome landscapes. This initiate a series of chromatin remodeling events that expose specific gene promoters and activate either pluripotency or cell type-specific gene regulatory networks (GRN) [8,9]. More recently, reprogramming of iPSCs and direct lineage conversion was also achieved with small molecules instead of exogenous transcription factors [10]. Although considerable progress has been achieved in understanding the GRNs for reprogramming, low efficiency [11], genetic/epigenetic instability [12,13], and in the case of direct lineage conversion, remnants of the initial cell fate, represent important issues that remain unsolved [14]. Moreover, several embryonic stem cell-specific *cis*-regulatory regions were not triggered during iPSC reprogramming [15]. Hence, there is a pressing need for identifying factors that can enhance reprogramming efficiency and maturation of converted cells, for example by taking cues from development [16] or through large-scale genetic screens [17,18]. Notably, accumulating evidence suggests a central role of non-coding RNAs, an important class of regulators of gene expression and chromatin remodeling, during development and cell fate specification, which has prompted scientists to elucidate the potential role of non-coding RNAs for iPSC reprogramming and direct lineage conversion.

In the first part of this review, we will discuss micro-RNAs (miRNAs), a class of non-coding RNAs, which have been shown to – either in combination with the forced expression of specific sets of transcription factors or on their own – modulate the reprogramming of fully differentiated mature cells into iPSCs or trans-differentiation into cells of a different lineage while bypassing an intermediate pluripotent state. In the second part, we will

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summarize the role of another class of non-coding RNAs, long non-coding RNAs (lncRNAs), in cell reprogramming. We rationalize that miRNAs and lncRNAs represent a promising and powerful tool for the future of reprogramming and regenerative medicine.

2. miRNAs: master orchestrators of gene expression

Since the discovery of the very first micro-(mi) RNA, lin-4, in *Caenorhabditis elegans* (*C.elegans*) [19,20], thousands of micro-(mi) RNAs have been identified in both, plants and animals, where they have the capacity to interact with RNA, DNA and protein molecules to regulate gene expression at multiple levels. The biogenesis and function of miRNAs have been comprehensively reviewed elsewhere [21,22]. Briefly, miRNAs are first transcribed by RNA polymerase II/III into long, primary transcripts called pri-miRNAs. Thereafter, pri-miRNAs are cut and processed into short pri-miRNAs of approximately 70–100 nucleotides in length which fold into a hairpin shape. This is mediated by a microprocessor complex consisting of a ribonuclease III protein called Drosha, a RNA-binding protein called DGCR3 and several other cofactors [23–26]. After export into the cytoplasm, either a RNase III called Dicer or an Argonaute protein, Ago2, converts pri-miRNAs into short, double-stranded miRNA molecules of approximately 22 nucleotide length [27,28]. One of the strands (guide-strand), which is complementary to its target, then is recruited by argonaute protein to form the miRNA-induced silencing complex (miRISC), with which the miRNA is usually guided to the 3′ UTR of its target mRNA. The other strand (passenger-strand), which is not complementary to target mRNA, has long been thought to be degraded during miRNA biogenesis [29], but there is mounting evidence that passenger strand miRNAs can also target mRNAs and exert functions for example during cancer formation [30,31]. However, in order to regulate gene expression, miRNAs not only bind to RNA, but they also have the potential to interact with DNA and protein molecules, which attributes them as master regulators in orchestrating GRNs [32–39].

2.1. The role of miRNAs in iPSC reprogramming

Gene regulatory networks (GRNs) that instruct developmental fates of cells are typically composed of specific sets of transcription factors, epigenetic modulators, signaling molecules and non-coding RNAs [40–43]. Advances in the field of cell reprogramming over the last decades have substantiated the importance of manipulating these GRNs in order to render different cell fates interchangeably [44–47]. Given the key role of miRNA in modulating GRNs, it is not surprising that manipulation of a variety of miRNAs has emerged as a potent method to either enhance or inhibit the reprogramming towards diverse cell types. Moreover, there is a growing number of miRNAs that have been shown to, on their own, have the capacity to reprogram cell fates. These findings underscore the importance of realizing the various functions of miRNAs in modulating GRNs, which will undoubtedly lead to the discovery of many more miRNAs with critical roles in cell reprogramming and cell fate decisions in coming years.

A common strategy for the identification of candidate miRNAs involved in iPSC reprogramming has been to analyze differently expressed miRNAs (profiled by means of microarrays or small RNA-sequencing) between pluripotent stem cells and differentiated cells during iPSC generation or during the differentiation into somatic cells [48–59]. An example of miRNAs that are downregulated during differentiation of ESCs and improve iPSC formation is a subset of the miR-290 cluster called the ES cell-specific cell cycle-regulating (ESCC) miRNAs, which includes miR-291-3p, miR-294 and miR-295 [60]. Introduction of miR-291-3p, miR-294 and miR-295 along with Oct-4, Sox2 and Klf4 (OSK) into mouse embryonic fibroblasts (MEFs) was found to consistently increase the number of Oct4-positive iPSC colonies compared with MEFs transduced with OSK alone [49]. Strongest improvement of iPSC formation was observed after introduction of miR-290, while other members of the same cluster, miR-292-3p and miR-293, had no effect. Interestingly, c-Myc binds to the promoter of these miRNAs, suggesting that they act downstream of c-Myc. In another study, mimics of human miR-302b and miR-372, which are orthologous to the mouse miR-291, miR-294 and miR-295, were able to enhance iPSC reprogramming efficiency [61]. While the subset of the miR-290 cluster described above was selected based on its expression during ESC differentiation, another study selected candidate miRNAs based on their upregulation during the early stages of iPSC reprogramming [51]. Overexpression of two members of the miR-106a cluster, miR-93 and miR-106b greatly enhanced iPSC generation, while knockdown of the same miRNAs as well as another member of the same cluster, miR-25, using miR-inhibitors decreased reprogramming efficiency. Further analyses revealed that both, miR-93 and miR-106a repressed TGFBR2 and p21 expression. Paradoxically, inhibiting TGFBRI kinases undermined ES cell renewal, whereas small molecule inhibitors of TGFBR1 enhanced iPSC reprogramming. Thus, factors that are essential for ESC self-renewal do not necessarily improve iPSC formation, but might even represent barriers for cell reprogramming.

Temporal gene expression profiling revealed that iPSC generation from MEF involves a multistep process characterized by initiation, maturation and stabilization phases [56]. In-depth analysis of the dynamic gene expression in combination with a systematic genetic RNAi screen revealed that the initiation phase is characterized by a coordinated mesenchymal-to-epithelial transition and BMP signaling, as well as upregulation of several miRNAs [56]. Inhibition of BMP signaling during the initiation phase suppressed the expression of multiple miRNAs including miR-200a, −200b, and −205, whereas exogenously supplied BMP enhanced their expression. Importantly, transfection of MEF with two miR-200 family mimics, Mim-200b and Mim-200c, in conjunction with OSKM, stimulated MET and accelerated through the initiation phase. Mechanistic insight into the synergism of members of the miR-200 cluster and OSKM during iPSC reprogramming revealed that Oct4 and Sox2 directly target the promoters of members of the miR-200 cluster and activate their expression [57]. Upon activation, miR-200 family members mainly targeted ZEB2 through directly binding to its 3′ UTR, thereby promoting mesenchymal-to-epithelial transition and enhancing iPSC generation.

In contrast to ESCC miRNAs, which are highly enriched in mouse ESCs (mESCs), miR-21 and miR-29a belong to the most abundant miRNAs in MEF [59]. Inhibition of their expression using miRNA inhibitors together with overexpression of the OSKM factors has been shown to enhance iPSC reprogramming efficiency [59]. c-Myc was found to play a predominant role in suppressing miR-21 and miR-29a at the transcriptional level. Analysis of the mechanism by which miR-21 and miR-29a affect reprogramming showed that both miRNAs inhibit expression of p53, which has a well-known role in modulating iPSC reprogramming [62–65]. Indeed, the p53 pathway, widely known for its role in tumor suppression, has recently emerged as a central roadblock for iPSC generation (reviewed in Ref. [66]). Although most of the targets of p53 are protein-coding genes, several miRNAs are vital components of the p53 pathway, which has prompted researchers to investigate their role during cell reprogramming. miR-34, miR-145 and miR199a, all of which are induced by p53, have been shown to inhibit iPSC generation via different mechanisms [67,68,58,69] (Fig. 1). Conversely, miR-138 has been shown to directly target p53, and several miRNAs, including miR-93, miR-106a/b and miR290, target

p21, a well-known component of the p53 pathway, thereby promoting iPSC generation [70,51,71]. Together with miR-21 and miR-29a, these miRNAs therefore constitute important modulators of the p53 GRN, locating it at the crossroads between cell reprogramming and tumor suppression.

Interestingly in a more recent study, miR-29a has also been reported to target DNA demethylases [72]. Repression of miR-29a led to the demethylation of fibroblast-specific DNA marks, and iPSC lines derived from miR-29a repression reduced the aberrant DNA methylation status observed in control-derived iPSCs and resembled human ESCs (hESCs) in their methylome. Conversely, another member of the miR-29 cluster, miR-29b, is upregulated by SOX2 and targets DNA methyltransferases (Dnmt3a and Dnmt3b), thereby enhancing iPSC formation [73]. Thus, two members of the same miRNA cluster, which both target epigenetic modulators, can have opposite roles during iPSC reprogramming. Notably, recent studies indicate that miR-29c, the third member of the miR-29 cluster, also targets Dnmt3a and Dnmt3b, indicating that miR-29c might also have a role during the formation of iPSCs, but this remains to be tested [74–76].

An alternative approach for the identification of candidate miRNAs is to perform high-throughput genetic screens [17,18]. Using MEFs carrying an Oct4-GFP reporter gene – which allows to measure the efficiency of iPSC generation by quantifying GFP expression – screened 570 chemically synthesized mouse miRNA mimics for their effect on iPSC reprogramming [17]. While validating previously known miRNAs, this screen identified miR-181, which was previously not associated with reprogramming, to promote iPSC generation. Notably, neither MEFs nor pluripotent stem cells express high levels of miR-181. Instead, miR-181 expression is transiently induced during the initial phase of iPSC reprogramming and subsequently silenced in iPSCs. A siRNA screen for targets of miR-181 and miR-294, an ESCC miRNA, furthermore revealed that during reprogramming initiation, the miR-181 and ESCC miRNA families have different targets, but come together when inhibiting TGF-β signaling and activating Wnt signaling, while ESCC miRNAs alone activated Akt signaling. In another screening study, several new miRNAs including miR130, miR-301 and miR-721 were found to enhance reprogramming of fibroblasts into iPSCs [18]. Analysis of target genes revealed that all three miRNA downregulated protein expression of the transcription factor Meox2 at the translational level. Transient silencing of Meox2 in MEFs significantly increased iPSC colony formation, albeit at lower levels than overexpression of the single miRNA, indicating that additional downstream effectors of these miRNA could have beneficial effects on iPSC reprogramming.

There are reports of miRNA that are able to reprogram iPSCs without additional pluripotency-associated transcription factors. For example, the miR302/367 cluster, which is highly expressed in ESCs and iPSCs, is composed of five miRNAs, four of which –miR302a/b/c/d-share an identical seed sequence [77]. The fifth miRNA, miR-367, has a different seed sequence and therefore is likely to target different mRNA transcripts. Lentiviral overexpression of miR302a/b/c/d was able to efficiently reprogram MEF and human fibroblasts to a pluripotent state in the absence of any additional pluripotent stem cell transcription factor [78]. Moreover, reprogramming using these miRNAs alone was significantly more efficient than with the previously identified OSKM factors. In the absence of miR-367, Oct4 expression was not induced, revealing that miR-367 activates Oct4 expression during reprogramming. Interestingly, in addition to the expression of miR-367, the HDAC inhibitor valproic acid (VPA) was essential for iPSC formation during reprogramming of MEF, while VPA was not required for the generation of iPSCs from human fibroblasts. VPA specifically degrades the HDAC2 protein, which is expressed at higher levels in MEF than in human fibroblasts. Thus, this study revealed that low levels of HDAC2 is critical during iPSC reprogramming, and that overexpression of miRNAs of the miR302/367 cluster without additional transcription factors required activation of Oct4 expression as well as epigenetic modulators. Similarly, combined transfection of miR200c, miR-302s and miR-369 was shown to be able to reprogram mouse and human somatic cells to pluripotency without additional transcription factors, albeit at very low efficiency (0.03%) [53]. Transfection of only one or two members in any combination did not generate iPSC colonies. Bisulfite sequencing revealed that CpG dinucleotides of the promoters of Nanog and Oct4 at later stages during reprogramming were less frequently methylated, indicating reactivation of immature status-related genes. Furthermore, in a recent study, knockout of miR-369 inhibited OSKM-mediated reprogramming of adipose-derived mesenchymal stem cells into iPSCs [79]. Overexpression of miR-369 was found to stabilize splicing factors of pyruvate kinase, which led to increased iPSC generation. If optimized further, the miRNA-only strategy will be of great interest for a clinical application, as it does not employ any integrating viral vectors.

2.2. miRNAs in transdifferentiation

Transdifferentiation, or direct cell reprogramming, refers to the direct conversion of a fully differentiated cell into another without passing through a pluripotent stage [80]. In the field of regenerative medicine, direct cell reprogramming is of particular interest due to the decreased risk of tumor formation and the potential to generate more homogenous cell populations with regard to reproducibility and subtype generation [81]. Neurons and cardiomyocytes are among the pivotal therapeutically relevant cell types, and several studies have addressed the possibility to directly convert differentiated cells into neurons or cardiomyocytes using either transcription factors, miRNAs, small molecules or a combination thereof (reviewed in Refs. [82,83]). While direct reprogramming of neurons and cardiomyocytes have been extensively discussed elsewhere [84,85,82,86–97,83,80,98,81], we will focus on studies that revealed different miRNAs with a role in mediating direct cell reprogramming towards neurons and cardiomyocytes.

Post-mitotic neural development and dendrite morphogenesis are mediated by repression of BAF53a by miR-9 and miR-124 [99]. Lentiviral overexpression of miR-9/9–124 along with the neurogenic factor NeuroD2 directly converted human fibroblasts into post-mitotic neurons expressing mature neuronal markers such as Map2 and firing action potentials [100]. Addition of two other neurogenic factors, Ascl1 and Myt1l, further increased maturation of the generated neurons and enhanced efficiency of direct conversion. During the process of neuronal differentiation, all subunits of the nBAF complex were upregulated (Baf53b, 45b and 45c), while miR-9/9–124 repressed Baf53a. Thus, miR-9/9–124 act on multiple targets during the reprogramming of human fibroblasts into induced neurons. In another study, combination of overexpression of miR-9/9–124 with transcription factors enriched in the developing striatum – BCL11B, DLX1, DLX2 and MYT1L-led to the direct conversion of human postnatal and adult fibroblasts into enriched population of striatal medium spiny neurons (MSN) [101]. Transfection of the striatal transcription factors without miR-9/9–124 was ineffective in generating neurons. After transplantation into the mouse brain, the induced MSN survived for up to 6 months and established typical connections to the anatomical targets of MSN. Indeed, miR-124 seems to take an important role in both, regulating normal neuronal differentiation as well as promoting conversion of various cell types into cells of the neuronal lineage.

For instance, miR-124 regulates the transcription silencing complex built on RE1-silencing transcription factor (REST), which in turn represses many neuronal-specific genes in non-neuronal cells, including miR-124 itself [102,103]. In a seminal study, suppression of polypyrimidine-tract-binding (PTB) protein, a target of miR-124, and its homolog nPTB in different cell types including HeLa cells, human embryonic carcinoma stem cells, mouse neuronal progenitor cells, human retinal epithelial cells and MEFs led to the direct conversion into functional neurons [104]. Subsequent studies revealed that PTB downregulation dismantles multiple components of the REST complex through different mechanisms, including direct competition with miRNA-targeting or boosting miRNA action on specific genes by changing local RNA secondary structure, thereby shielding or enhancing miRNA target sites in adjacent regions. Since components of the REST complex show several polypyrimidine-tract-binding (PTB) protein binding sites which are previously validated target sites of miR-124, miR-9 and miR-449 [105,106], these results suggest that PTB downregulation enables dismantling of the REST complex by multiple components of the miRNA machinery. As a result, critical REST-repressed transcription factors such as Ascl1, Brn2, Zic1, Olig2, and NeuroD1 – previously shown to induce transdifferentiation of MEFs into neurons [107] – were upregulated.

In another example of miRNAs' involvement in transdifferentiation, transient transfection of mouse fibroblasts with miR-1, miR-33, miR-208 and miR-499 (miR-combo) was able to convert them into functional cardiomyocytes [108]. Addition of the small molecule JAK inhibitor I drastically enhanced this process, while other compounds tested had no beneficial effect. Furthermore, intramyocardial injection of the same miRNAs immediately after injury by permanent ligation of the left ascending coronary artery revealed conversion of cardiac fibroblasts into cardiomyocytes in vivo. Notably, the same group identified a specific media composition consisting of advanced DMEM/F12, ascorbic acid and insulin-transferrin-selenium, which increased conversion efficiency of the miR-combo by up to 15 fold [109]. More recently, another study revealed that addition of miR133a to the transcription factors Gata4, Mef2c and Tbx5 improved cardiac reprogramming of mouse and human fibroblasts through down-regulation of Snai1, a master regulator of epithelial to mesenchymal transition [110]. Inclusion of miRNA133a significantly increased the number of beating induced cardiomyocyte-like cells and shortened the duration until the appearance of beating cells.

However, miRNAs have also proven to be essential for the direct reprogramming of other cell types other than neurons and cardiomyocytes. Transfection of astrocytes with viral particles

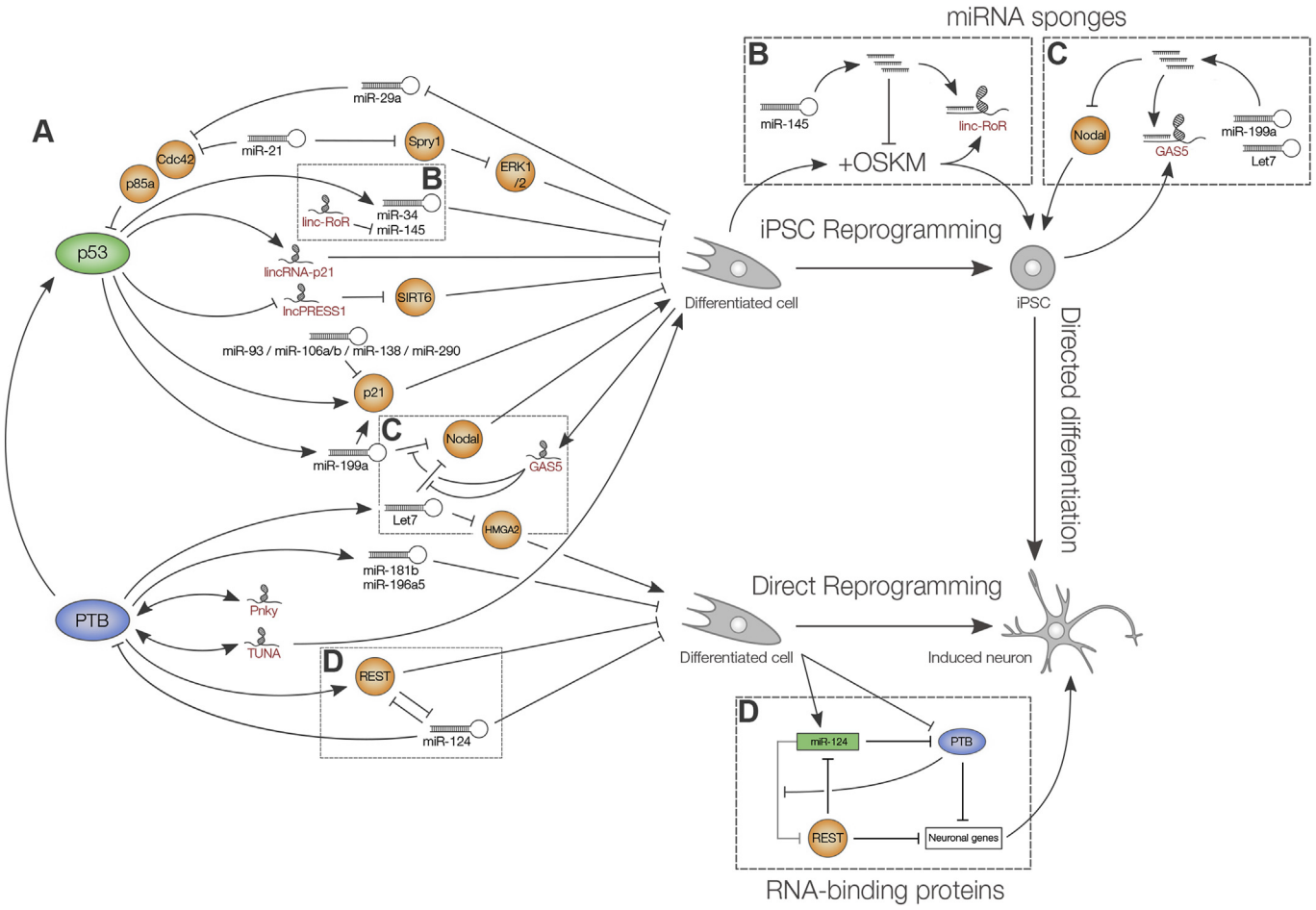

Fig. 1. Non-coding RNA-centered Gene Regulatory Network (GRN) in cell reprogramming. (**A**) A simplified schematic summarizing miRNAs and lncRNAs with known roles in modulating GRNs involved in cell reprogramming downstream of p53 and PTB. (**B** + **C**) The lncRNAs linc-RoR (B) and GAS5 (C) act as competitive inhibitors termed "miRNA sponges", containing multiple tandem binding sites for miRNAs which inhibit cell reprogramming. (**D**) During development, miR-124 promotes neuronal differentiation by targeting REST, while being inhibited by REST in a feedback loop. The RNA-binding protein PTB suppresses targeting of REST by multiple miRNAs including miR-124. REST and PTB ultimately converge in repressing many neuronal genes, and either overexpression of miR-124 or downregulation of PTB enhances direct reprogramming of fibroblasts into neurons.

expressing miR-302/367 has been shown to directly convert human astrocytes into neuroblasts [111]. This conversion required addition of VPA. Importantly, during a 2 month follow up study, no tumor formation of the converted neuroblasts was observed, making this approach promising for the treatment of various neurological diseases. In a different study, overexpression of miR302/367 together with miR9/9–124 in human fibroblasts led to the direct conversion into neurons [112]. The converted neurons upregulated synaptic markers in vitro and in vivo, indicating establishment of connectivity. Interestingly, several miRNAs including miR-302 have also been shown to facilitate conversion of pancreas progenitor cells. Co-transfection of miR-302 with PDX1, NGN3 and MAFA into a human hepatic cell line activated expression of pancreas progenitor cell-related gene expression, leading to the generation of insulin and C-peptide producing cells when combined with a chemically defined medium [113]. Thus, miR-302 may represent a more general miRNA facilitating the indirect reprogramming of various cell types into pluripotent cells or the direct conversion into different somatic cells.

Taken together, research over the last years has enlightened the great potential of miRNAs to regulate several gene regulatory networks involved in cell reprogramming. Further elucidation of the diverse functions of miRNAs during iPSC reprogramming and transdifferentiation will not only enhance our understanding of the underlying mechanisms, but also accelerate the establishment of cell reprogramming in regenerative medicine.

3. Long non-coding RNAs

The Encyclopedia of DNA Elements (ENCODE) and the Functional Annotation of the Mammalian Genome (FANTOM) projects have shown that the majority of products of the mammalian genome are long non-coding RNAs (lncRNAs) with yet uncharacterized functions [114–116]. Usually defined as non-protein coding transcripts longer than 200 nucleotides, lncRNAs are transcribed by RNA polymerase II and further capped and spliced, similar to coding mRNA. Hence, from a biochemical point of view, lncRNAs are not distinct from mRNA. However, lncRNAs lack an open reading frame (ORF) and a large proportion of them are not poly-adenylated. Until now, lncRNAs have been shown to function by modulating the transcriptome via the regulation of epigenetic, transcriptional, and post-transcriptional mechanisms [117]. lncRNAs acts as (i) signals, mirroring transcriptional processes and hence may represent markers of biological events; (ii) decoys for miRNA target sites which titrate away miRNAs and regulatory proteins; (iii) guides that recruit chromatin remodeling factors/enzymes in *cis* or *trans*; and (iv) scaffolds, enabling the formation of ribonuclear protein complexes involved in gene regulatory events [118,119]. Thus, the intracellular localization of lncRNAs within chromatin, sub-nuclear domains, the nucleoplasm or the cytoplasm can provide vital clues to their mechanism of action [120]. Furthermore, lncRNAs have been shown to be poorly conserved when compared to other small non-coding RNAs, suggesting that the secondary structure may represent their main functional unit [121].

3.1. LncRNAs as additional regulatory layer in reprogramming and pluripotency

Pluripotency-associated networks of genes, including the core transcription factors and aforementioned miRNAs, govern the pluripotent state [45]. Precise understanding of this regulatory network is essential for somatic cell reprogramming so as to modulate the epigenetic state of somatic cell towards pluripotency. Recent evidence indicates that lncRNAs function as an additional regulatory layer in maintaining pluripotency and determining cell fate [122–129,45,130,131]. Thus, lncRNAs may form additional nodes in the gene regulatory network (GRN), either as upstream elements for maintaining pluripotency/cell fate conversion or as alternative paths for reprogramming. Delineating the regulatory role of lncRNAs in core GRNs controlling the acquisition of pluripotency or mediating cell fate conversion requires comprehensive understanding of lncRNA expression profiles during the conversion of pluripotent cells to a defined fate or during reprogramming of somatic cells into iPSCs. In this section, we will focus on the role of lncRNAs in reprogramming, and subsequently provide examples of lncRNAs that have been shown to modulate cell fate conversion to the neural lineage.

3.2. LncRNAs and reprogramming

Lineage-committed cells are characterized by distinct regulatory epigenetic marks that safeguard cellular identity [132]. Therefore, derailing this epigenetic barrier is the prime event initiating cell fate conversion. Forced transformations of cell fates, by manipulating expression of pluripotency/lineage-related transcription factors, results in drastic epigenetic modifications coupled with widespread transcriptomic changes [16,9]. The regulatory potential of lncRNAs in governing epigenetic and transcriptomic changes is well established, and growing evidence attributes lncRNAs as an important regulator during cell reprogramming.

While characterizing the transcriptional dynamics during iPSC formation, a study by Loewer et al. revealed 10 lncRNAs that are involved in achieving pluripotency [133]. Among them, regulator of reprogramming lncRNA (lincRNA-RoR) emerged as an important candidate whose perturbation resulted in altered reprogramming efficiency of fibroblasts to iPSCs [133]. The mechanistic role of lincRNA-RoR was subsequently characterized, where it acts as a key competing endogenous RNA for the network of miRNAs regulating core transcription factors involved in pluripotency. Most importantly, lincRNA-RoR shares seed sequences with miR-145, the miRNA that represses expression of core pluripotency transcription factors [134,69]. iPSCs express lincRNA-RoR at a higher level than miR-145, thus enabling it to shield miR-145 target sites and preventing miRNA-mediated repression.

Later, a large intergenic noncoding RNA p21 (lincRNA-p21) was identified, which impaired reprogramming by sustaining H3K9me3 and CpG methylation at pluripotency gene promoters [135]. While, lincRNA-p21 is induced by p53 during reprogramming, it does not promote apoptosis or cellular senescence, but interacts with methyltransferases SETDB1 and DNMT1 through the RNA-binding protein HNRNPK to maintain the repressive chromatin state [135]. LncPRESS is another p53-induced lncRNA crucial for maintenance of ESC identity. This particular lncRNA physically interacts with the histone deacetylase SIRT6 and restricts its chromatin localization, resulting in high levels of histone H3K56 and H3K9 acetylation at pluripotency gene promoters. Depletion of lncPRESS1 induced lineage-specific genes with concurrent reduction in expression of pluripotency genes [136].

Once pluripotency is achieved, lncRNAs also regulate proliferation and self-renewal of iPSCs. Initial studies have attributed lncRNAs in the maintenance of pluripotency based on their co-expression with pluripotency related genes [137]. In hESCs, a pioneering study revealed three lncRNAs (AK056826, EF565083, BC026300) that are exclusively expressed in undifferentiated hESCs/iPSCs and co-expressed with key pluripotency drivers [138], and depletion of these lncRNAs promoted differentiation.

Later, systematic *loss-of-function* experiments identified several lncRNAs that regulate core pluripotency transcription factors. Utilizing a genome-wide shRNA screen of mESC transcripts together with chromatin immunoprecipitation of key pluripotency

transcription factors, the lncRNAs Rncr2/AK028326 (Oct4-activated) and AK141205 (Nanog-repressed) were identified to maintain pluripotency [139]. *Rncr2*/AK028326 furthermore was shown to act as a co-activator of Oct4 in a regulatory feedback loop [139]. Another genome-wide siRNA/shRNA screen in mESCs revealed additional 26 lncRNAs regulating the pluripotent state, which was validated using a Nanog luciferase reporter system [140], and *Panct1, Panct2*, *Panct3* (AK081885) to regulate mESC identity [141]. *Panct1* was followed up for depletion studies, which led to downregulation of pluripotency related gene expression, but this study lacked mechanistic explanation for *Panct1* function. A more recent large scale 'loss of pluripotency' screen, which employed CRISPR interference (CRISPRi) targeting 16,401 lncRNAs in iPSCs, indicated that inhibition of only 9 lncRNA loci reduced POU5F1/OCT4 expression [142].

Multiple studies revealed that lncRNAs regulate pluripotency by a variety of mechanisms including histone modifications, as scaffolds and by forming competing endogenous RNA (ce-RNA) for miRNAs that repress expression of pluripotency genes. Apart from the evidences of co-expression with pluripotency-related genes, Dinger et al. identified developmentally regulated lncRNAs in mESCs named Evx1as and Hoxb5/6as that physically interacted with tri-methylated H3K4 histones and histone methyltransferase MLL1, suggesting epigenetic mechanisms that coordinate pluripotency and differentiation [137]. Notably, lncRNA also regulate H3K27 methylation at promoters of differentiation-related genes, resulting in their transcriptional repression. H3K27 di/trimethylation involves the polycomb repressive complex 2 (PRC2), and the ARID domain-containing protein (JARID2) forms an essential accessory factor for the proper recruitment of PRC2 [143]. Several lncRNA encoded within the imprinted Dlk1-Dio3 locus were reported to bind to JARID2. Of these, MEG3 binding promoted recruitment and assembly of PRC2 at differentiation-related genes - maintaining the pluripotency of hiPSCs [144].

A recent study revealed that transition from pluripotency to differentiation required downregulation of ribosomal RNA synthesis in the nucleolus, resulting in ribosomal DNA heterochromatinisation [145]. The lncRNA pRNA was shown to interact with nucleolar repressor factor TIP5 to promote both H3K27 and H3K9 tri-methylation during differentiation of mESCs. Thus, depletion of pRNA exemplified a role of lncRNA modulating pluripotency through regulation of chromatin remodeling, retaining ESCs in a pluripotent state exhibiting a reduced number of heterochromatic loci.

LncRNAs can also function as scaffolds to maintain pluripotency by promoting recruitment of transcription factors and other chromatin modifiers [136]. Recently, human endogenous retrovirus subfamily H (HERVH) transcripts were identified to function as lncRNAs, forming a scaffold which recruits p300 and OCT4 to HERVH LTR7 regions to regulate pluripotency-associated transcripts [146]. Additionally, Tcl1 Upstream Neuron-Associated lincRNA (TUNA) functions as a scaffolding-lncRNA, forming a complex with RNA-binding proteins that binds to the promoters of pluripotency drivers [147]. Recently, a lncRNA - AK048794 was also shown to act as competing endogenous ce-RNA for miR-592, resulting in de-repression of its endogenous target, FAM91A1, which is involved in maintenance of pluripotency [148]. Likewise, growth-arrest-specific transcript 5 (GAS5), which is highly expressed in hESCs and whose expression correlated with hESC self-renewal, functions as ce-RNA, reducing targeting of miRNA to the TGF beta receptor family ligand NODAL, thereby sustaining NODAL expression and promoting hESC self-renewal [149]. Taken together, these reports revealed a complex regulatory network of lncRNAs that orchestrates acquisition and maintenance of pluripotency.

3.3. LncRNAs involved in neural fate decision: evidences from ESCs and iPSCs

Embryonic neural development is characterized by the expression of numerous lncRNAs whose role in promoting neural differentiation has been well characterized [150]. Although the large number of spatio-temporally expressed lncRNAs underscores their functionality, the underlying regulatory mechanisms of many of them remain elusive, demanding for large-scale perturbation studies to characterize their function [142].

In one comprehensive study pertaining to neural fate determination of hESCs, Ng et al. identified a group of 35 lncRNAs involved in differentiation of hESCs into neurons. Mechanistic studies revealed that these lncRNAs physically interacted with the nuclear proteins REST and SUZ12 [138]. In a subsequent study, one of these lncRNAs, rhabdomyosarcoma 2-associated transcript (*RMST*), was shown to form an indispensable regulatory complex required for SOX2 binding to the promoter regions of neurogenic transcription factors, thereby co-regulating downstream genes implicated in neurogenesis [151]. In another study, depletion of *TUNA* resulted in transition from pluripotency towards a neural cell fate [147]. Mechanistically, *TUNA* formed a complex with three RNA-binding proteins, PTBP1, hnRNP-K, and Nucleolin, regulating key pluripotency-associated genes Nanog, Sox2, and Fgf4 [147]. In another example, *HOTAIRM1*, a *cis*-acting regulator of the HOXA gene cluster and CRNDE, located in proximity to the IRX5 gene which is involved in brain patterning and neurogenesis, were upregulated in human iPSCs that differentiated into neurons [152]. Furthermore, a recent report revealed that the loss of non-coding RNA expression form the DLK1-DIO3-imprinted locus correlated with reduced neural differentiation potential of hESC lines [153]. Together, these studies revealed a large number of lncRNAs that are differentially expressed during the differentiation of hiPSCs to neurons, but comprehensive and systematic characterization of their function is still lacking.

Notably, lncRNAs that regulate direct cell reprogramming have not been identified yet. The abundance of lncRNAs in the mammalian genome demands for large-scale perturbation studies during direct cell programming coupled with molecular phenotyping and functional characterization. Given the prominent role of lncRNAs in epigenetic control and the fact that transdifferentiation involves epigenetic modifications, such studies are likely to reveal lncRNAs involved in direct reprogramming of different cell types including neurons [125,154].

4. Future perspectives

Discovery of the increasingly diverse characteristics of miRNA and lncRNAs requires a deeper mechanistic exploration into localization, structure, and interacting partners to decipher their involvement in cell reprogramming. Additional studies that use large-scale transcript perturbation techniques such as CRISPR/Cas9 will provide an unprecedented advantage for functionally characterizing novel non-coding RNAs [142]. Moreover, single-cell transcriptome analysis in ESCs/IPSCs have been employed to reveal dynamically expressed miRNA and lncRNAs at different stages of reprogramming [155,156]. With rapid reduction in cost for sequencing, the single-cell resolution to study gene expression profiling will allow highly accurate and efficient isolation of target cell types, making it suitable technology for both basic and clinical stem cell research. As more mechanisms of non-coding RNAs are revealed, additional opportunities to devise techniques to generate high quality iPSCs and directly converted cell types will become prevalent – accelerating the application of reprogrammed cells in the clinics.

Declaration of interest

Authors claim no conflict of interest.

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