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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