PROSPECTS
Journal of Cellular Biochemistry 114:275–281 (2013)

Journal of Cellular Biochemistry

MicroRNA-Mediated Somatic Cell Reprogramming

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ABSTRACT

Since the first report of induced pluripotent stem cells (iPSCs) using somatic cell nuclear transfer (SCNT), much focus has been placed on iPSCs due to their great therapeutic potential for diseases such as abnormal development, degenerative disorders, and even cancers. Subsequently, Takahashi and Yamanaka took a novel approach by using four defined transcription factors to generate iPSCs in mice and human fibroblast cells. Scientists have since been trying to refine or develop better approaches to reprogramming, either by using different combinations of transcription factors or delivery methods. However, recent reports showed that the microRNA expression pattern plays a crucial role in somatic cell reprogramming and ectopic introduction of embryonic stem cell-specific microRNAs revert cells back to an ESC-like state, although, the exact mechanism underlying this effect remains unclear. This review describes recent work that has focused on microRNA-mediated approaches to somatic cell reprogramming as well as some of the pros and cons to these approaches and a possible mechanism of action. Based on the pivotal role of microRNAs in embryogenesis and somatic cell reprogramming, studies in this area must continue in order to gain a better understanding of the role of microRNAs in stem cells regulation and activity. J. Cell. Biochem. 114: 275–281, 2013.

KEY WORDS: MicroRNA; miR-302; miR-367; miR-291/294/295; EMBRYONIC-LIKE STEM CELL; STEM CELL REPROGRAMMING; iPSC; EMBRYONIC STEM CELL-SPECIFIC MicroRNA; EPIGENETIC MODIFICATION; CYCLIN-DEPENDENT KINASE

The recent advances in understanding somatic cell reprogramming have brought the stem cell closer to fulfilling its great clinical promise. Initially, cellular differentiation was thought to occur only in a unidirectional manner, but studies done in the last decade have demonstrated that somatic cell nuclear transfer (SCNT) could lead to a reprogramming event [Amano et al., 2001; Wilmut et al., 2007]. Subsequently, researchers have used SCNT to produce animal clones from numerous species. This method, though, requires an oocyte due to the crucial role played by the maternal cytoplasmic components in development, impeding the use of SCNT for clinical purposes. In addition, the complexity associated with SCNT suggests that it may be unsuitable for clinical applications that require the mass production of reprogrammed cells. Takahashi and Yamanaka [2006] developed a groundbreaking methodology to induce reprogramming in mice fibroblasts using the four defined factors, Oct4, Sox2, Klf4, and c-Myc. The same group then confirmed this finding in human fibroblasts and found that the embryonic stem cell-like colonies that originate from the fibroblasts display characteristics similar to human embryonic stem cells [Takahashi et al., 2007]. Importantly, these studies demonstrated the feasibility of producing induced pluripotent stem cells (iPSCs) from somatic cells, circumventing the use of human embryo, oocyte, or any associated embryonic materials, and therefore avoiding ethical controversies that could greatly hinder research progress. Also, this technique allows for the production of patient-specific iPSCs, which dramatically reduces the potential of immune rejection due to the fact that the resultant iPSCs and host cells carry the same genetic information. A problem with this technique, though, is that these defined factors have tumorigenic properties. Although excluding c-Myc or using different combinations of reprogramming factors, such as Oct4, Sox2, Nanog, and Lin28, has been shown to reduce tumorigenicity [Yu et al., 2007; Huangfu et al., 2008], these modified methods resulted in a reprogramming efficiency that is too low for large production.

Numerous reports have described the pivotal role of specific miroRNAs (miRNAs) in reprogramming. Suh et al. [2004] found that the microRNA expression pattern in human embryonic stem cells (ESCs) greatly differs from that of differentiated cells, and identified a group of microRNAs abundantly expressed in human ESCs. These microRNAs, termed embryonic stem cell-specific microRNAs, include miR-302a, miR-302b, miR-302c, miR-302d, miR-367, and miR-371-373. Among them, the expression of the miR-302/367 cluster in human ESCs peaks before differentiation and then dramatically declines after differentiation. These findings spurred

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Manuscript Received: 11 July 2012; Manuscript Accepted: 30 August 2012
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 7 September 2012
DOI 10.1002/jcb.24385 • © 2012 Wiley Periodicals, Inc.

scientists to study the potential use of embryonic stem cell-specific microRNAs in somatic cell reprogramming. Indeed, several studies showed that miR-302/367 greatly improved the efficiency of four factor-mediated reprogramming while the knockdown of proteins involved in microRNA biogenesis significantly decreased reprogramming efficiency [Liao et al., 2011; Li et al., 2011]. Furthermore, several recent reports have described the successful use of various combinations of microRNAs and delivery methods to reprogram various cell lines [Anokye-Danso et al., 2011; Miyoshi et al., 2011].

MicroRNAs BIOGENESIS AND FUNCTIONS

MicroRNAs are a type of small non-coding RNAs, usually consisting of 19–25 nucleotides, which regulate gene expression post-transcriptionally by mediating the cleavage or translational repression of targeted mRNAs [Bartel, 2004; Ying et al., 2006; Carthew and Sontheimer, 2009]. The canonical biogenesis of miRNA starts with the transcription by RNA polymerase II in the nucleus to produce primary microRNAs (pri-microRNAs). Pri-microRNAs form unique single strand and double strand conjunctions that are then recognized by the RNA-specific endonuclease Drosha and the microprocessor subunit DGCR8. This results in the cleavage of pri-microRNA to generate the precursor transcripts of microRNA (pre-microRNAs) [Han et al., 2006]. Alternatively, additional proposed microRNA biogenesis routes have been reported in which endonucleases other than Drosha process the pri-microRNA [Yang and Lai, 2011]. The proposed Drosha-independent pathways comprise different intermediates, including mirtrons that are short intronic hairpins; tailed mirtrons that may be processed by exosomes or an unspecified nuclease, depending on the tail position; tRNA–shRNA fusions, and; endo-siRNA. Regardless of the intra-nuclear processing of the microRNA, Ran-GTP/Exportin-5 recognizes the single strand on the 3′ end of pre-microRNA and then subsequently transports it out of the nucleus into the cytosol for further cleavage. In the cytoplasm, Dicer, a type III ribonuclease, cleaves the pre-microRNA into 19–25 bp-long hairpin RNA duplexes, which then associate with Argonautes and other accessory proteins to form the RNA-induced silencing complex (RISC) [Hammond et al., 2001; Czech and Hannon, 2011]. Within the microRNA:RISC complex, the seed sequence on the 5′ untranslated region (UTR) of the microRNA recognizes the 3′ UTR of the targeted mRNA and, based on sequence complementarity, this may result in either target degradation, or translation suppression [Fabian et al., 2010]. Because the targeting of microRNA does not require perfect pairing between the two sequences, a single microRNA may target multiple mRNAs that have similar sequences on their 3′ ends, and conversely, more than one microRNA may target a single mRNA.

REGULATORS AND TARGETS OF THE MIR-302/367 CLUSTER

The miR-302/367 cluster is encoded in human chromosome 4 and consists of miR-302a, miR-302a*, miR-302b, miR-302b*, miR-302c, miR-302c*, miR-302d, miR-367, and miR-367*. These nine members are poly-cistronic and co-transcribed from the same promoter, which is targeted by the homedomain proteins Oct4, Sox2, Nanog, and Rex1 [Deng et al., 1995]. Studies in multiple cell types have demonstrated a positive correlation between expression of miR-302 and Oct4, Nanog, and Sox2 [Barroso-del Jesus et al., 2008, 2009]. In addition, it has been found that expression of miR-302a and Oct4 occurs at the same stages and in the same tissues during embryonic development.

Recent efforts to identify miR-302/367 targets have helped elucidate the reprogramming mechanism [Chih-Hao and Shao-Yao, 2012]. Among the cluster, miR-302a, miR-302b, miR-302c, and miR-302d share the same seed sequence on the 5′ UTR and therefore have overlapping targets [Barroso-del Jesus et al., 2009]. Lin and et al. showed that the miR-302 family targets four epigenetic regulators, which include two AOF family members, AOF1 and AOF2, as well as MECP1-p66 and MECP2. AOF1 and AOF2 silence gene expression through the demethylation of histone 3 on lysine 4 (H3K4). Downregulation of AOF2 correlates with decreased DNMT1 expression levels, and thus miR-302 also has an indirect effect on DNA methylation [Lin et al., 2011]. MECP1-p66 and MECP2 are important epigenetic regulators that bind to specific methylated regions of DNA, and an independent study has confirmed that miR-302b targets MECP2 [Subramanyam et al., 2011]. The same study reported that the human orthologs hsa-miR-302b and hsa-miR-372 target multiple molecules involved in cellular processes other than epigenetic regulation, such as the cell cycle, epithelial–mesenchymal transition (EMT), and even vesicular transport. They also observed an enhanced efficiency of defined transcription factor-mediated reprogramming after inhibiting the EMT molecules RHOC and TGFBR2 [Subramanyam et al., 2011]. Recently, a combination of analytical techniques showed that, in hESCs, miR-302/367 promotes bone morphogenetic protein (BMP) signaling through the repression of its inhibitors, TOB2, DAZAP2, and SLAIN1 [Lipchina et al., 2011]. Trophectodermal fate is also promoted in response to the down-regulation of BMP. Of interest, BMP plays an important role in the induction of the mesenchymal-to-epithelial transition (MET), which is proposed to serve as the initiation phase of reprogramming in mouse embryonic fibroblasts [Samavarchi-Tehrani et al., 2010].

MiR-302/367 targets many cell cycle proteins, including the well-known G1–S transition cell cycle regulators cyclin D1, and CDK2. The ectopic expression of miR-302a causes the translational inhibition of cyclin D1 and thus results in an accumulated population of primary and malignant cells in the S phase and a decreased population of cells in the G1 phase, which resembles the cell cycle profile of embryonic stem cells [Card et al., 2008]. A subsequent study showed that the miR-302 family inhibits cell proliferation by promoting several G1–S transition arrest pathways through the silencing of cyclin D1/D2, CDK2, and BMI-1 [Lin et al., 2010]. An illustration is provided to summarize the downstream targets of miR-302 cluster in Figure 1.

Of note, the miR-291/294/295 cluster, a mouse analogue of human miR-302, seems to have a different function in mESC as it has been previously reported to cause fast cell proliferation rather than G1–S transition quiescence by targeting p21Cip1 [Deng et al., 1995].

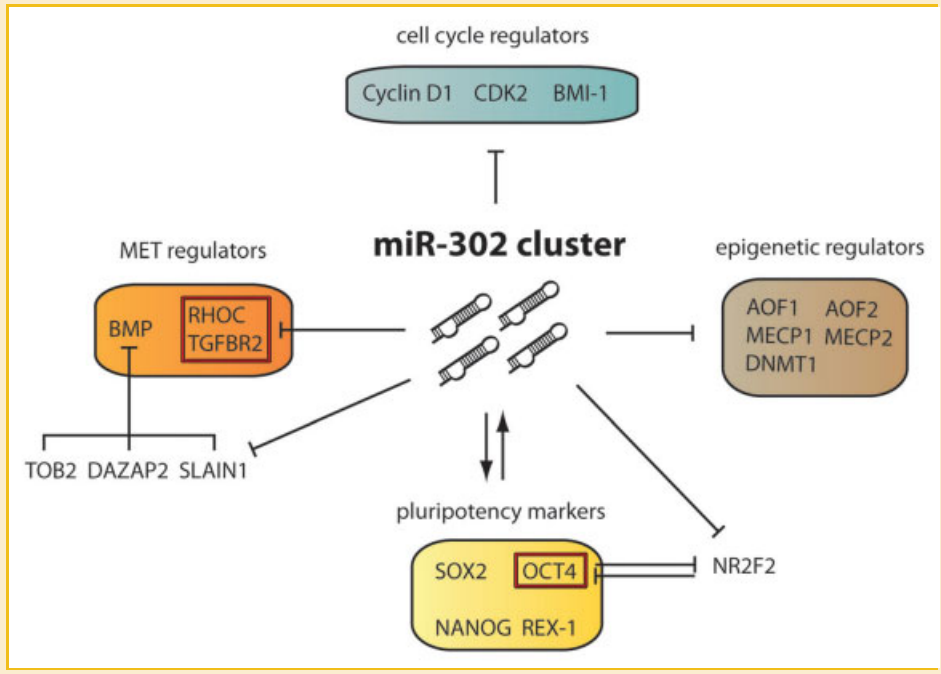

Fig. 1. Targets of miR-302 cluster. MiR-302 cluster affects cell cycle profile by targeting important cell cycle regulators such as Cyclin D1, CDK2, and BMI-1. AOF1, AOF2, MECP1, and MECP2 are also inhibited by ectopic expression of miR-302 family. This results in reduced stability of DNMT1 and global de-methylation. The silencing effect of miR-302 cluster on NR2F2 increases the level of OCT4, which, in return, further elevates the expression of miR-302 by binding to its promoter region. Together with global de-methylation, this reciprocal effect increases the expression of other pluripotency factors such as SOX2, NANOG, and REX-1. MiR-302 also induces MET (mesenchymal-to-epithelial transition) by targeting EMT promoters RHOC and TGFBR2 and, TOB2, DAZAP2, and SLAIN1 which are inhibitors of BMP, a MET promoter.

POTENTIAL MECHANISM FOR SOMATIC CELL REPROGRAMMING

DNA methylation determines the specific expression pattern in cells and plays an essential role in mammalian development. A prerequisite for somatic cell reprogramming is the removal of DNA methylation on the promoter regions of crucial embryonic stem cell transcription factors, including Oct4, Nanog, and Sox2. Once the genome is "unveiled," the transcription machinery can gain access to these genes and further activate their expression to initiate the reprogramming process. As described previously, miR-302 targets the epigenetic regulators that are responsible for different types of DNA methylation. Among these targets, AOF1, also known as KDM1b or LSD2, is an important histone H3K4 and H3K9 demethylase that is essential for the de novo DNA methylation which is required to establish maternal genomic imprints in oocytes [Ciccone et al., 2009]. A study examining the role of AOF2, which is another lysine-specific histone demethylases belongs the same family as AOF1, in mice showed that deficiency in its expression leads to arrested embryonic development at or before E5.5. The same AOF2 gene deficiency in ES cells induces DNA demethylation, thereby, inhibiting the capacity for differentiation. In addition, a negative correlation exists between expression of the DNA methyltransferase 1 (DNMT1) protein and AOF2, possibly due to the instability of DNMT1 in AOF2-depleted ES cells [Wang et al., 2009]. The same result was observed in human hair follicle cells (hHFCs) in which the overexpression of miR-302 downregulates AOF2 protein translation [Lin et al., 2011]. Taken together, the evidence indicates that miR-302 downregulates AOF1 and AOF2, leading to global DNA demethylation; an effect that is further enhanced by the miR-302-mediated DNMT deficiency and, decreased MECP1 and MECP2 expression.

Somatic cell reprogramming begins with the resetting of the genomic DNA methylation pattern. This alteration in methylation causes the gene expression profile to resemble that of embryonic stem cells by granting transcription factors, such as Oct4, Sox2, and Nanog, access to the DNA.

Lin et al. [2011] used SCNT to seek further experimental evidence of a connection between microRNA activity and somatic cell reprogramming (Fig. 2). Instead of transferring somatic cell nuclei into enucleated oocytes, they injected cell nuclei from human fibroblasts into enucleated iPSCs induced by ectopic miR-302 expression and observed embryoid bodies in more than 90% of the hybrid cells. Moreover, using bisulfite DNA sequencing, they detected DNA demethylation patterns on the promoter regions of Oct4 and Nanog similar to those in human embryonic stem cells H1 and H9. However, transferring the nuclei of miR-302-induced iPSCs into somatic cell cytoplasm did not generate embryoid bodies. Together, these findings imply that the potential reprogramming factors reside in the cytoplasm of the miR-302-induced iPSCs rather than in the nucleus, which is identical to conventional SCNT using oocyte cytoplasm.

Since the materials in the cytoplasm are the key to DNA demethylation and reprogramming, it is unlikely that Oct4, Sox2, and Nanog initiate this event because they are all nuclear transcription factors. Nevertheless, the up-regulation of these

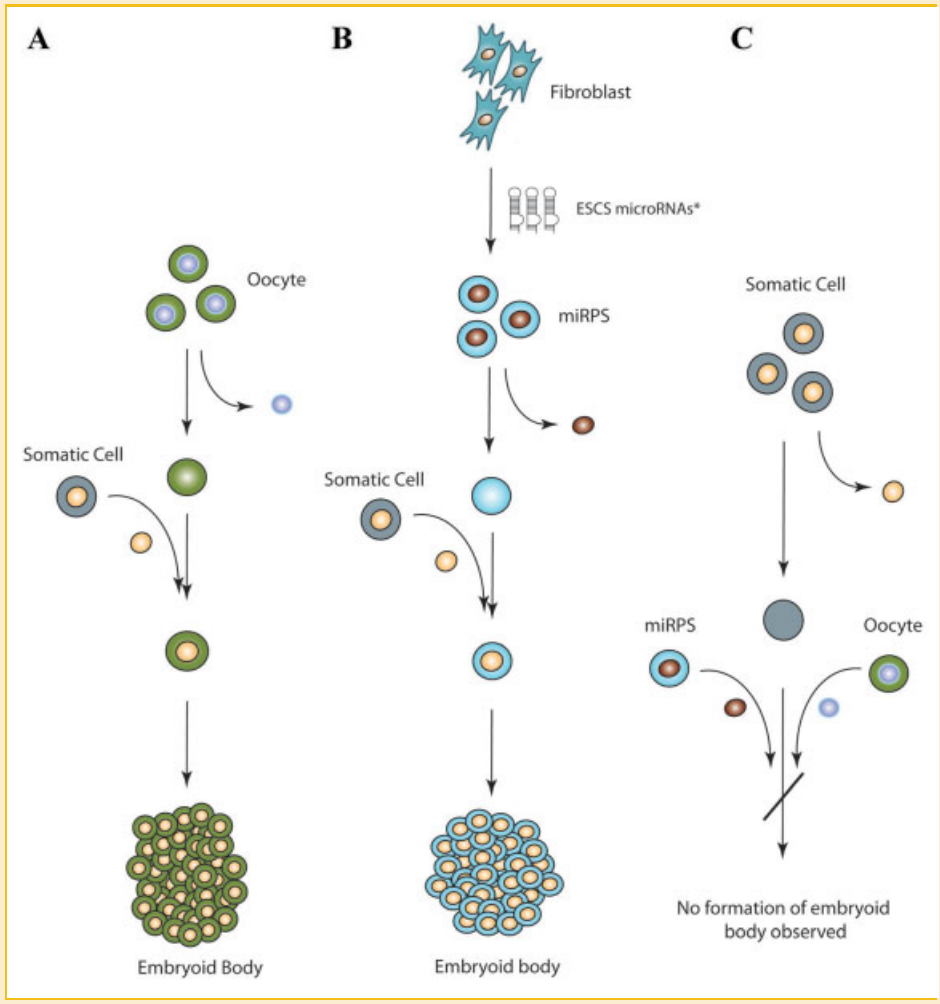

Fig. 2. Schematic illustration of three somatic cell nuclear transfer (SCNT) approaches. A: In traditional SCNT, the nucleus of an unfertilized egg is removed and the egg subsequently implanted with the nucleus extracted from a somatic cell. The reconstructed cell is capable of multiplication, and the formation of embryoid body can be observed after 5–7 days. B: An embryoid body can, too be derived by injecting the nucleus of a human fibroblast cell (hFB) into an enucleated mircroRNA-induced pluripotent stem (mirPS) cell. C: The fusion of cytoplasm from an enucleated somatic cell with the nucleus of a mirPS cell does not result in the formation of an embryo body after implantation.

defined nuclear factors is still considered a critical step in reprogramming and maintaining pluripotency. The question then remains as to how miR-302 interacts with Oct4, Sox2, Nanog, and Lin28 to orchestrate reprogramming. A group of researchers in New York reported that miR-302 directly target NR2F2, a member of a nuclear receptor subfamily that negatively regulates Oct4 and is expressed during differentiation [Rosa and Brivanlou, 2011]. Exogenous induction of miR-302 inhibits protein translation of NR2F2 by directly targeting its mRNA at the transcriptional level. A reduction of NR2F2 together with the removal of the genomic methylation site on the Oct4 promoter due to global DNA demethylation results in an increase in Oct4 expression. As described earlier, Oct4, and Sox2 transcriptionally activate the expression of the miR-302 cluster by binding to its promoter region [Card et al., 2008]. As a consequence, an increase in cellular Oct4 levels promotes the expression of the miR-302 cluster and other transcription factors, such as Sox2 and Nanog. This positive reciprocal loop is the driving force for the formation of iPSCs.

The evidence indicates that other miRNAs contribute to reprogramming. For example, Yu et al. [2007] reported that Oct4, Sox2, Nanog, and Lin 28 facilitate reprogramming; Lin 28 is an ESC-specific RNA binding protein that interacts with and suppresses the activity of let-7 miRNAs [Newman et al., 2008]. These observations suggest that the inhibition of miRNA may facilitate the canonical reprogramming approach. Subsequently, it was shown that c-Myc-enhanced reprogramming is partly due to the repression of MEF-enriched miRNAs, including miR-21 and miR-29a [Yang et al., 2011]. miR-34 miRNAs were recently identified as p53 targets that play an essential role in restraining somatic reprogramming [Choi et al., 2011]. The miRNA family miR-130/301/721 enhances iPSC generation via repression of Meox2 [Pfaff et al., 2011] while the miR-200s family (miR-200a, miR-141, and miR429) and miR-205 may contribute to stress-induced senescence [Cufi et al., 2012]. Furthermore, factors such as hypoxia-inducible factor (HIF) can induce an hESC-like transcriptional program, including the induced pluripotent stem cell (iPSC) inducers, Oct4, Nanog, Sox2, Klf4, cMyc,

and microRNA-302 in multiple cancer cell lines [Mathieu et al., 2011]. These findings point to the potential interaction between miRNAs and proteins, and the complexity of miRNA-based reprogramming.

Recent efforts to elucidate the molecular mechanism of pluripotency have yielded important information; however, none of the defined transcription factor studies have clearly defined how the factors modulate genome-wide DNA demthylation, the prerequisite step for successful reprogramming. For a cell to regain pluripotency, it has to bypass several barriers, including the removal of epigenetic modifications, activation of essential pluripotent genes and the induction of mesenchymal-to-epithelial transition (Fig. 3). Increasing experimental evidence supporting the effect of microRNA on epigenetic regulation, cell cycle regulation, and mesenchymal-to-epithelial transition, as well as its interaction with core embryonic stem cell specific transcription factors illustrates the central role of microRNA in reprogramming (Fig. 2). However, numerous reports indicate that reprogramming efficiency can be substantially improved by introducing additional reprogramming factors, such as SV 40 large antigen (SV40LT) and human telomerase reverse transcriptase (hTERT) to the four Yamanaka factors (OSKM) [Park et al., 2008]. Even the addition of SV40 alone with Oct4, Sox2, Nanog, and Lin28 dramatically increased the reprogramming efficiency, by up to 70-fold [Mali et al., 2008]. Additionally, a recent study even showed that Vitamin C enhances the quality of somatic cell reprogramming [Esteban and Pei, 2012].

REPROGRAMMING OF CANCER CELLS

Over the past decade, emerging evidence indicates a possible relationship between somatic cell reprogramming and tumorigenicity. For example, Miyoshi et al. [2010] reported the successful reprogramming of several gastrointestinal cancer cell lines after simultaneously introducing different combinations of iPSC transcription factors Oct3/4, Sox2, Klf4, and c-Myc, as well as oncogenes (BCL2 and KRAS), and tumor suppressor gene shRNAs (TP53, P16 (INK4A), PTEN, FHIT, and RB1). The expression of these transcription factors induced the GI cancer cells to develop an "immature status," defined as the activation of promoter regions of embryonic stem cell specific-defined factors, such as Nanog and Oct3/4. Indeed, these cells, referred to as induced pluripotent stem cell-like cancer cells (iPCCs), express significantly higher levels of endogenous Nanog mRNA as compared with parental cells, as well as express several embryonic stem cell-specific markers. At the same time, the iPCCs down-regulate expression of the tumor suppressor gene P16 (INK4A), but up-regulate expression after extended culture on a gelatin-coated plate. The same study showed that the iPCCs have a decrease in methylation at the Nanog promoter, accounting for the increase in Nanog expression and changes in epigenetic modifications may also account for the reactivation of P16 expression. Their findings strongly support the importance of epigenetic changes in cell tumorigenicity. In fact, epigenetic alterations appear as important as genetic mutations in the development of cancer cell characteristics [Hahn and Weinberg, 2002]. As discussed previously, the removal of parental epigenetic modifications, such as DNA methylation, serves as the first step in the reversal of differentiated somatic cells to the pluripotent state, and a number of reports have shown that miR-302 as well as other microRNAs play crucial roles in the remodeling of epigenetic patterns, indicating a potential role for microRNAs in cancer therapy [Lin et al., 2011; Li et al., 2012].

The effect of embryonic stem cell-specific microRNAs in tumorigenicity has also been extensively studied. Recently, it was shown that miR-302 might suppress the p53-signaling pathway by targeting PTEN, which co-operates with p53 in tumor suppression [Lipchina et al., 2012]. Deregulation of the cell cycle has been long recognized as the primary characteristic of cancer cells. The regulation of cell cycle transitions is tightly controlled by the activity of various cyclin–CDK complexes, such as cyclin E-CDK2 for the G1–S transition, cyclin A-CDK2 for the S-phase progression, cyclin D-CDK4/6 for the G1 progression. Various cyclin-dependent

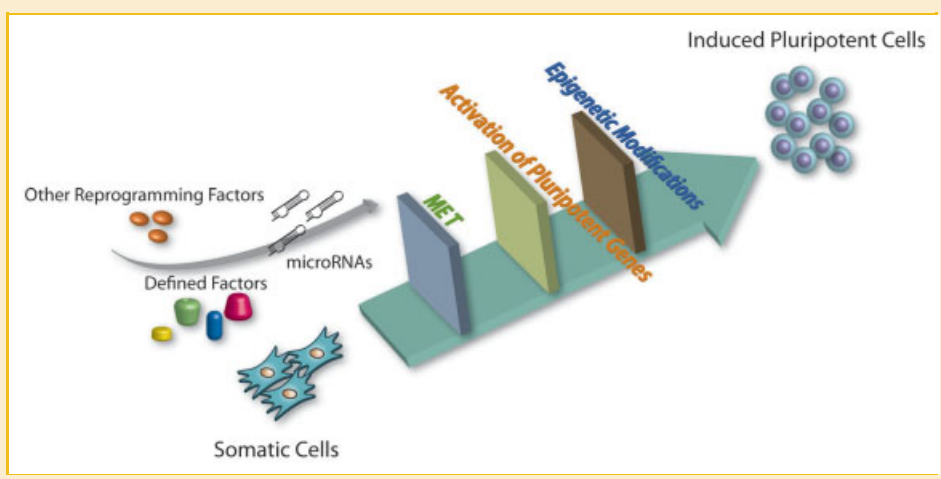

Fig. 3. The road to pluripotency. Successful reprogramming requires three essential events: Initiation of the mesenchymal–to–epithelial transition; removal of epigenetic modifications for the transcription of pluripotent genes; and subsequent activation of transcriptional and translational machinery for the expression of pluripotent genes.

kinases often regulate these cell cycle controllers. Notably, cyclin-dependent kinase inhibitor 1 or CDKN1A has been identified as a target of the miR-291/294/295 family, which is the mouse ortholog of human miR-302. In one study, the ectopic expression of any of the miR-291/294/295 family members promotes embryonic stem cell proliferation [Wang et al., 2008]. In contrast, another study showed that human miR-302 seems to suppress tumorigenicity in microRNA-mediated induced pluripotent stem cells through the simultaneous suppression of CDK2, cyclin D1/D2, and the cancer stem cell marker BMI-1, leading to inhibition of the G1–S phase transition [Lin et al., 2010]. It is possible that these conflicting experimental outcomes result from differences in species-specific functionality. Although the exact underling mechanism is still not well-understood, it is clear that microRNAs play pivotal roles in the control of cell proliferation and epigenetic modification.

CONCLUSION AND FUTURE PERSPECTIVES

Reprogramming of somatic cells into iPSCs by using transcription factors usually occurs at low efficiency, however, emerging evidence has shown that embryonic stem cell-specific miRNAs enhance reprogramming efficiency. More importantly, ESC-specific miRNAs alone can achieve successful reprogramming, suggesting that these miRNAs play an important role in the process. The primary advantage of miRNAs is that, unlike transcription factors, they directly and immediately alter the adult transcriptome and proteome, leading to increased efficiency and decreased time for inducing cell re-direction. Interestingly enough, ESC-specific miRNAs target various cell cycle regulators and tumor suppressors that not only induce pluripotent stem cells, but also alter cancer cell growth. The mechanism by which ESC-specific miRNAs re-direct somatic and cancer cells to gain pluripotency remains unknown. Nevertheless, miRNAs-based reprogramming could prove to be useful for the refinement of current reprogramming techniques and may provide new strategies not only in regenerative medicine and developmental disorder diseases, but also for cancer therapeutics.

ESC-specific miRNA-mediated reprogramming may start a new era in medicine and biotechnology. First, they are highly conserved and may control the spatial and temporal expression of genes crucial for fine-tuning of signaling pathways in early embryonic development. To date, increasing evidence suggests that miRNAs play a critical role in regulating the response to DNA damage. It is intriguing to elucidate the role that ESC-specific miRNAs play by interacting with proteins in the DNA instability and methylation/demethylation during reprogramming and/or development. Second, given that there has been a consistent observation of upregulation of the ESC-specific miR-302 cluster and related miRNAs during reprogramming, the possibility exists that miRNAs can be used as ESC markers for monitoring the reprogramming process. Third, miR-371-373 and miR-302 clusters are universally overexpressed in malignant germ cell tumors (GCTs) and coordinately downregulate mRNAs involved in biologically significant pathways, suggesting that these miRNAs are markers for GCTs. Furthermore, the miR-302 cluster is found in several glioma and medullobalstoma cells lines and the miR 302-367 cluster drastically affects self-renewal and infiltration properties of glioma-initiating cells (also known as cancer stem cells). Together with the fact that miRNAs are stable and their levels can be measured in the saliva and blood as well as that there are numerous similar characteristics between ESCs and cancer stem cells (CSC), suggests that ESC-specific miRNAs may prove useful as novel putative cancer stem cell markers and therapeutic targets. Fourth, a regulatory circuit exists among OCT4, miR-302, and NR2F2: miRNA-302 and OCT4 reciprocally feedback to one another and NR2F2 directly inhibits OCT4. Thus, determination of the optimal miR-302 level in the cytosol to initiate and maintain reprogramming would shed light on the control of reprogramming, allowing for the optimization of this technique. Fifth, studies have suggested that miRNAs may play important roles in regulating self-renewal and differentiation of skin stem cells. It may be beneficial to determine whether the perpetual production of miR-302 in the cytosol of reprogrammed cells can be used as a therapeutic strategy for repairing skin damage. Finally, ESC-specific miRNA target sites exist in the transcripts encoding disordered proteins, which may be involved in age-related diseases, and as such, ESC-specific miRNAs may hold important clues in elucidating the aging process. In summary, there is little doubt that a better understanding of miRNA-based reprogramming will help elucidate mechanisms underlying development, tumor growth, and disorder-related aging.

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